Programmed DNA Damage in Myogenesis

A thesis submitted to the National University of Ireland in fulfilment of the requirement for the degree of Doctor of Philosophy.

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Head of Department: Prof John Kelly

October 2016.
Acknowledgements

Foremost, I would like to thank my supervisor Dr Howard Fearnhead. I owe him a debt of gratitude for his patience, enthusiasm, and immense knowledge. His guidance made this project into a success. I could not have imagined having a better teacher and mentor for these last four years.

I would also like to express my gratitude to my graduate research committee: Dr Declan McKernan, Dr Maura Grealy, and Prof John Kelly. Our intense GRC meetings kept me on the right track.

I thank my good friends and lab mates in the Fearnhead group, past and present: Jonathan Doran, Aoife Nolan, Daria Firsova, Amin Tashakor, Mahshid Dehkordi, and Alireza Noori. I would also like to thank my friends from the wider lab, particularly the PCI group. A special thanks must go to Dr Alessandro Natoni, upon whose endless knowledge and patience I, and many others, have been dependent.

To all of the friends I have made over these last few years: I thank you all for reminding that there exists a world outside the laboratory!

Finally, I would like to thank my family; particularly my two young nephews, Nate and Jack.

Patrick F Connolly
26/10/2016
Abstract

Skeletal muscle is a major tissue type in humans, comprising approximately 40% of total body mass in adults. Skeletal muscle is ultimately composed of elongated multinucleate cells known as myofibers. These myofibers are formed through the differentiation and cell fusion of individual myoblast cells. This process of myogenic fusion is the basis of muscle development in utero, and of muscle regeneration.

Myogenic fusion requires the activity of caspases, the proteolytic effectors of apoptotic cell death. One outcome of this myogenic caspase activity is the generation of genomic DNA strand breaks. This occurs through the activity of the endonuclease CAD (caspase-activated DNase). The function of these DNA strand breaks in the context of myogenic differentiation is still unknown. I hypothesize that the role of these strand breaks is to activate elements of the myogenic genetic program by way of the DNA damage response, perhaps through kinase-mediated activation of myogenic transcription factors.

Here, I show that artificially-induced DNA strand breaks produced by an exogenous source are sufficient to induce the differentiation of myoblasts in vitro. I also show that a key DNA damage response kinase, DNA-PK, is required for the myogenic differentiation program. This is in line with the theory that DNA damage response factors are involved in propagating the myogenic signal. Furthermore, I show that inhibition of several cell cycle checkpoint kinases induces spontaneous myogenic differentiation, accompanied by massive genomic DNA strand breakage, again suggesting that DNA strand breakage is sufficient to induce differentiation.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ac-DEVD-AMC</td>
<td>N-Acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin</td>
</tr>
<tr>
<td>ADAP</td>
<td>Adhesion and degranulation-promoting adapter protein</td>
</tr>
<tr>
<td>APAF-1</td>
<td>Apoptotic protease activating factor 1</td>
</tr>
<tr>
<td>ATM</td>
<td>Ataxia telangiectasia mutated</td>
</tr>
<tr>
<td>ATR</td>
<td>Ataxia telangiectasia and Rad3 related</td>
</tr>
<tr>
<td>BAI1</td>
<td>Brain-specific angiogenesis inhibitor 1</td>
</tr>
<tr>
<td>Bax</td>
<td>Bcl-2-associated X protein</td>
</tr>
<tr>
<td>Bcl</td>
<td>B-cell lymphoma</td>
</tr>
<tr>
<td>BID</td>
<td>BH3 interacting-domain death agonist</td>
</tr>
<tr>
<td>BrdU</td>
<td>Bromodeoxyuridine</td>
</tr>
<tr>
<td>CaCl</td>
<td>Calcium chloride</td>
</tr>
<tr>
<td>CAD</td>
<td>Caspase-activated Dnase</td>
</tr>
<tr>
<td>CARD</td>
<td>Caspase activation and recruitment domain</td>
</tr>
<tr>
<td>CCD</td>
<td>Charge-coupled device</td>
</tr>
<tr>
<td>CDC</td>
<td>Cell division cycle protein</td>
</tr>
<tr>
<td>CDK</td>
<td>Cyclin-dependent kinase</td>
</tr>
<tr>
<td>CED</td>
<td>Cell death abnormality</td>
</tr>
<tr>
<td>CHAPS</td>
<td>3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate</td>
</tr>
<tr>
<td>CHK1</td>
<td>Checkpoint kinase 1</td>
</tr>
<tr>
<td>DDR</td>
<td>DNA damage response</td>
</tr>
<tr>
<td>DED</td>
<td>Death effector domain</td>
</tr>
</tbody>
</table>
DIDS 4,4'diisothicyanato-stilbene-2,2'-disulfonic-acid
DISC Death-Inducing Signaling Complex
DM Differentiation medium
DMC1 DNA Meiotic Recombinase 1
DMEM Dulbecco's modified eagle medium
DMSO Dimethyl sulfoxide
DNA-PK DNA-dependent protein kinase
DSB Double-stranded DNA break
DTT Dithiothreitol
EDTA Ethylenediaminetetraacetic acid
EdUTP 5'-Ethynyl-2'-deoxyuridine 5'-triphosphate
EGFR Epidermal growth factor receptor
EGTA Ethylene glycol-bis(β-aminoethyl ether)-tetraacetic acid
EtOH Ethanol
FBS Fetal bovine serum
GAS-2 Growth arrest-specific protein 2
GM Growth medium
H2AX Histone 2AX
HBSS Hank's balanced salt solution
HEPES 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IC50 Half-maximal inhibitory concentration
ICAD Inhibitor of caspase activated Dnase
ICE Interleukin-1β-converting enzyme
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Full Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>KCl</td>
<td>Potassium chloride</td>
</tr>
<tr>
<td>MGI-2</td>
<td>Macrophage and Granulocyte Inducer type 2</td>
</tr>
<tr>
<td>MMS</td>
<td>Methyl methanesulfonate</td>
</tr>
<tr>
<td>MRF4</td>
<td>Myogenic regulatory factor 4</td>
</tr>
<tr>
<td>MyHC</td>
<td>Myosin heavy chain</td>
</tr>
<tr>
<td>MyoD</td>
<td>Myogenic differentiation protein</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
</tr>
<tr>
<td>NaOH</td>
<td>Sodium hydroxide</td>
</tr>
<tr>
<td>NHEJ</td>
<td>Non-homologous end-joining</td>
</tr>
<tr>
<td>PARP</td>
<td>Poly ADP ribose polymerase</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PCD</td>
<td>Programmed cell death</td>
</tr>
<tr>
<td>PEI</td>
<td>Polyethylenimine</td>
</tr>
<tr>
<td>PKB</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>PUMA</td>
<td>p53 upregulated modulator of apoptosis</td>
</tr>
<tr>
<td>RAG</td>
<td>Recombination Activating Genes</td>
</tr>
<tr>
<td>ROCK-1</td>
<td>Rho-associated, coiled-coil-containing protein kinase 1</td>
</tr>
<tr>
<td>RPA</td>
<td>Replication protein A</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse-transcription polymerase chain reaction</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small-interfering RNA</td>
</tr>
<tr>
<td>SSB</td>
<td>Single-stranded DNA break</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>SYK</td>
<td>Spleen tyrosine kinase</td>
</tr>
<tr>
<td>TdT</td>
<td>Terminal deoxynucleotidyl transferase</td>
</tr>
<tr>
<td>TE</td>
<td>Tris-EDTA</td>
</tr>
<tr>
<td>TMRE</td>
<td>Tetramethylrhodamine, ethyl ester</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>TRAIL</td>
<td>TNF-related apoptosis-inducing ligand</td>
</tr>
<tr>
<td>TUNEL</td>
<td>Terminal deoxynucleotidyl transferase dUTP nick end labeling</td>
</tr>
<tr>
<td>TX-100</td>
<td>Triton-X-100</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra-violet</td>
</tr>
<tr>
<td>VDAC</td>
<td>Voltage-dependent anion channel</td>
</tr>
<tr>
<td>XIAP</td>
<td>X-linked inhibitor of apoptosis</td>
</tr>
</tbody>
</table>
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Figure 1: EGFR inhibitors block myogenic differentiation
Materials & Methods
Materials & Methods

Materials

*Commonly-used reagents for buffers and biochemical assays*

Agarose, Sigma-Aldrich, St. Louis, MO
Boric acid, Sigma-Aldrich, St. Louis, MO
Bradford reagent, Sigma-Aldrich, St. Louis, MO
Calcium chloride (CaCl), Sigma-Aldrich, St. Louis, MO
Dithiothreitol (DTT), Sigma-Aldrich, St. Louis, MO
Ethanol, Sigma-Aldrich, St. Louis, MO
Ethylenediaminetetraacetic acid (EDTA), Sigma-Aldrich, St. Louis, MO
Ethylene glycol-bis(β-aminoethyl ether)-tetraacetic acid (EGTA), Sigma-Aldrich, St. Louis, MO
Glycerol, Sigma-Aldrich, St. Louis, MO
N-Acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin (Ac-DEVD-AMC), Sigma-Aldrich, St. Louis, MO
N-laurylsarcosine, Sigma-Aldrich, St. Louis, MO
Protease inhibitor cocktail, Sigma-Aldrich, St. Louis, MO
Phenylmethanesulfonyl fluoride (PMSF), Sigma-Aldrich, St. Louis, MO
Phosphatase inhibitor cocktail, Sigma-Aldrich, St. Louis, MO
Phosphate-buffered saline (PBS), Sigma-Aldrich, St. Louis, MO
Potassium chloride (KCl), Sigma-Aldrich, St. Louis, MO
Resazurin dye, Sigma-Aldrich, St. Louis, MO
Sodium chloride (NaCl), Sigma-Aldrich, St. Louis, MO
Sodium hydroxide (NaOH), Sigma-Aldrich, St. Louis, MO
Tris base, Sigma-Aldrich, St. Louis, MO
3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), Sigma-Aldrich, St. Louis, MO
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), Sigma-Aldrich, St. Louis, MO

Enzymes and associated products
Restriction enzymes, New England Biolabs, Ipswitch, MA
RNase A, Sigma-Aldrich, St. Louis, MO

Drugs compounds
Tocris kinase inhibitor toolbox (a full list of compounds used in this screen is detailed in Annex III), Tocris Biosciences, Bristol, UK
CHIR-124, Selleckchem, Houston, TX
Embryomax nucleoside mixture (containing cytidine, guanosine, uridine, thymidine, and adenosine), Merck-Millipore, Billerica, MA
KU-55933, Selleckchem, Houston, TX
MK-1774, Selleckchem, Houston, TX
NU-7026, Selleckchem, Houston, TX
NU-7441, Selleckchem, Houston, TX
PD-407824, Selleckchem, Houston, TX
Purvalanol B, Tocris Biosciences, Bristol, UK
Q-VD-OPh, ApexBio, Houston, TX
Materials for DNA preparation, purification, transfection, and PCR

Acetone, Sigma-Aldrich, St. Louis, MO
Ampicillin, Sigma-Aldrich, St. Louis, MO
Chloramphenicol, Sigma-Aldrich, St. Louis, MO
Chloroform, Sigma-Aldrich, St. Louis, MO
Forward and reverse primers, Origene, Rockville, MD
Isopropanol, Sigma-Aldrich, St. Louis, MO
Kanamycin, Sigma-Aldrich, St. Louis, MO
LB agar, Sigma-Aldrich, St. Louis, MO
LB medium, Sigma-Aldrich, St. Louis, MO
Polyethylenimine (PEI), Polyscience, Warrington, PA
Qiagen Miniprep Kit, Qiagen Inc., Valencia CA
Qiagen OneStep RT-PCR kit, Qiagen Inc., Valencia CA
siRNA constructs, Origene, Rockville, MD
Sybr Safe, Invitrogen, Waltham, MA
Tri reagent, Sigma-Aldrich, St. Louis, MO
5X Colourless GoTaq Flexi loading buffer, Promega, Madison, WI
**Immunocytochemistry and immunoblotting**

Anti-mouse antibody conjugated to Alexa Fluor 488, Molecular Probes, Eugene, OR

Anti-Myosin Heavy Chain antibody (MF-20), Developmental Studies Hybridoma Bank, Iowa City, IA

Click-iT TUNEL Alexa Fluor 588 Imaging Assay, Molecular Probes, Eugene, OR

Fetal bovine serum (FBS), Sigma-Aldrich, St. Louis, MO

Hoechst 33342, Sigma-Aldrich, St. Louis, MO

IRDye 800CW or 680RD secondary antibodies, Licor Biosciences, Lincoln, NE

Nitrocellulose membrane, Sigma-Aldrich, St. Louis, MO

Non-fat milk, Sigma-Aldrich, St. Louis, MO

Paraformaldehyde, Sigma-Aldrich, St. Louis, MO

Phosphate-buffered saline (PBS), Sigma-Aldrich, St. Louis, MO

Poly-D-lysine-treated glass coverslips, Sigma-Aldrich, St. Louis, MO

SDS-PAGE loading buffer, Sigma-Aldrich, St. Louis, MO

SDS-PAGE molecular weight ladder, Sigma-Aldrich, St. Louis, MO

Sodium dodecyl sulfate (SDS), Sigma-Aldrich, St. Louis, MO

Tris base, Sigma-Aldrich, St. Louis, MO

Triton X-100, Sigma-Aldrich, St. Louis, MO

**Mammalian and bacterial cell lines**

C2C12 myoblasts, Sigma-Aldrich, St. Louis, MO

DH5-Alpha *E. coli* cells, gifted from another lab
Cell culture reagents and materials

Dulbecco’s Modified Eagle Medium (DMEM), Sigma-Aldrich, St. Louis, MO
Fetal bovine serum, Sigma-Aldrich, St. Louis, MO
Hank’s Balanced Salt Solution (HBSS), Sigma-Aldrich, St. Louis, MO
Horse serum, Sigma-Aldrich, St. Louis, MO
Penicillin/Streptomycin, Sigma-Aldrich, St. Louis, MO
Trypsin-EDTA, Sigma-Aldrich, St. Louis, MO

Equipment

CytoFluor Series 4000 fluorimeter, Perceptive Applied Biosystems, Waltham, MA
NanoDrop spectrophotometer, NanoDrop Products, Wilmington, DE
Odyssey CLx point-source laser scanner, LI-COR Biosciences, Lincoln, NE
Pharos FX Plus laser gel scanner, Bio-Rad, Hercules, CA
Philips TUV G30T8 UV-C mercury discharge lamp. Philips, Amsterdam, Netherlands
Veriti Gradient Thermal Cycler, Applied Biosystems, Waltham, MA
Victor 3V 1420 spectrometer, Perkin Elmer, Waltham, MA

Software

ImageJ, National Institutes of Health, Bethesda, MD
GNU Image Manipulation Program (GIMP), GNOME Foundation, Orinda, CA
Graphpad Prism, GraphPad Software Inc, CA
Methods

*C2C12 cell culture & differentiation*

Mouse C2C12 myoblasts (Sigma-Aldrich, St. Louis, MO) were suspended in Dulbecco’s Modified Eagle Medium (DMEM, Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum and 1% Penicillin/Streptomycin (Sigma-Aldrich, St. Louis, MO); henceforth referred to as Growth Medium or GM. To induce differentiation, the cells were seeded into tissue culture plates at $5 \times 10^4$ cells cm$^{-2}$ and incubated at 37°C in 5% CO$_2$ atmosphere. After 24h, the cells were washed three times with Hank’s Balanced Salt Solution (HBSS, Sigma-Aldrich, St. Louis, MO), and cultured in DMEM supplemented with 2% horse serum and Penicillin/Streptomycin (Differentiation Medium; DM). Differentiation was marked by fusion of cells into elongated myotubes, and was typically allowed to proceed for 3 days, at which point the maximum level of fusion is attained.

*Quantitation of myotube formation*

Cells differentiated on tissue culture plates or poly-D-lysine-treated glass coverslips were washed to remove non-adherent cells and fixed in 4% paraformaldehyde at room temperature for 20 minutes. Cells were permeabilized (0.1% Triton X-100 for 15 minutes) and blocked with 10% FBS for 1 hour before being stained with anti-Myosin Heavy Chain antibody (MF-20, Developmental Studies Hybridoma Bank, Iowa City, IA; 1:1000) overnight and detected with a secondary goat anti-mouse antibody conjugated to Alexa Fluor 488 (Molecular Probes, Eugene, OR; 1:1,000 for 1 hour). Nuclei were counterstained with Hoechst 33342 (2 µg/mL) for 20 minutes. Myotubes were visualized under inverted epifluorescence microscopy and images captured using a CCD. The extent of fusion was quantified using the fusion index, where the number of nuclei
found within myotubes (defined as syncytia containing two or more nuclei) was expressed as a percentage of the total number of nuclei recorded per field.

**Drug treatments**

Cells were seeded in tissue culture plates and cultured in DM or GM supplemented with drug compounds or 0.01% DMSO for the duration of the experiment. A full list of all drugs used in this study can be found in the Materials section and in Annex III.

**Resazurin cell viability assay**

Cells were grown and differentiated in 96 well plates. At the endpoint, the differentiation medium was removed and replaced fresh DM. This was supplemented with resazurin dye (Sigma-Aldrich, St. Louis, MO) to a final concentration of 93 µM. The plates were incubated for 6 hours at 37 °C in 5% CO₂. Dye reduction was measured using a Victor 3V 1420 spectrometer (λex 530 nm, λem 595 nm).

**Caspase activity assay**

Adherent cells were harvested by trypsinization. Cells were centrifuged at 400 × g for 5 minutes, before being resuspended in lysis buffer (50 mM HEPES, pH 7.4, 10 mM KCl, 2mM MgCl₂, 5 mM EGTA, 0.1% CHAPS, 100 µM PMSF, and 1 mM DTT), and incubated on ice for 15 minutes. Protein concentrations were determined by Bradford assay and were then normalized. A fluorogenic caspase-3 substrate DEVD-AMC (Sigma-Aldrich, St. Louis, MO) diluted in lysis buffer was added to a concentration of 10 µM changes in fluorescence (λex 380 nm and λem 450 nm) over time detected using a Perceptive Biosystems CytoFluor Series 4000 fluorimeter. The rate
of enzymatic activity was determined from the linear phase of the time-course, and was reported as AFU·min⁻¹·mg total protein.

Methods for assessing DNA damage

- TUNEL assay

Cells were differentiated on poly-D-lysine-treated glass coverslips, washed to remove non-adherent and apoptotic cells and fixed with 4% paraformaldehyde for 15 minutes at room temperature, followed by permeabilization with 0.25% Triton X-100 in PBS for 20 minutes at room temperature. TUNEL staining was carried according to the manufacturer’s instructions (Click-iT TUNEL Alexa Fluor 588 Imaging Assay, Molecular Probes, Eugene, OR). Briefly, cells were incubated with TdT enzyme and EdUTP nucleotide mixture, and incubated for 120 minutes at 37 °C to label free DNA ends. An Alexa Fluor 488 fluorophore-based probe was then conjugated to the terminal EdUTP using a 3-6 a copper (I) catalysed click reaction. Nuclei were counterstained with Hoechst 33342 and visualized under inverted epifluorescence microscopy and images were recorded using a CCD. TUNEL staining was reported as percent positive nuclei per field.

- Single cell gel electrophoresis (comet) assay

Cells were grown, treated, and differentiated in 6 well tissue culture plates. The cells were harvested and washed once in PBS. The cells were combined with low-melting point agarose at a 1:10 ratio, then pipetted onto a microscope slide. The slide was chilled at 4 C for 10 minutes to fully solidify the agarose. The slide was immersed for 30 minutes in chilled lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris base, 1% N-laurylsarcosine, and 0.5% Triton X-100). The slides were then transferred to chilled alkaline unwinding solution (0.3 M NaOH, 10 mM EDTA)
and incubated for 20 minutes. After this, the slides were equilibrated in TBE for 5 minutes, then subject to electrophoresis at 35V (4.375 V/cm) for 20 minutes. The slides were submerged in diH2O for 5 minutes, then chilled 70% EtOH was added dropwise to fix the DNA. After 5 minutes, the EtOH was removed, and the slides allowed to air dry completely. The slides were stained with SYBR Green in TE buffer for 20 minutes, then washed once in TE for 5 minutes. Images were recorded using an inverted epifluorescence microscope in the FITC channel (490 nm). The length of each comet tail (µm) was measured using ImageJ (National Institutes of Health, Bethesda, MD).

**UV-irradiation of cells**

Cells were grown in standard 24 well tissue culture plates (Sarstedt, Nümbrecht, Germany). At the time of irradiation, the volume of culture medium in each well was 1 mL. The plates were positioned in a tissue culture hood at a fixed distance from a UV-C germicidal lamp (Philips TUV G30T8, power output: 12W, peak emission: 250 nm). The power density at the culture hood surface was estimated to be 117 µW/cm². After irradiation, the cells were returned to the 37°C incubator.

**Immunofluorescence**

Cells were differentiated in tissue culture plates. Cells were washed twice with PBS. Optionally, cells were pre-extracted with 0.5% Triton X-100 in PBS with 100 µg/mL RNase A for 30 minutes at room temperature. Cells were fixed in 4% paraformaldehyde in PBS for 20 minutes at room temperature. Cells were then permeabilized with 0.2% Triton X-100 for 20 minutes at room temperature. The cells were blocked with 10% FBS in PBS for 1 hour at room temperature. Following this, the cells were incubated with the appropriate antibody overnight at room
temperature on an orbital shaker. After washing, the cells were probed using Alexa Fluor fluorescent secondary antibodies (typically 1:1,000 dilution, Molecular Probes, Eugene, OR) at room temperature for 1 hour. The cells were counterstained with Hoechst 33342 at 2 µg/mL for 20 minutes, before being visualized using inverted epifluorescence microscopy. Images were recorded using a CCD.

**Western blotting**

Cell monolayers were trypsinized and collected by centrifugation. The cells were resuspended in RIPA lysis buffer supplemented with protease and phosphatase inhibitor cocktails (Sigma-Aldrich, St. Louis, MO). The suspensions were vortexed vigorously and an aliquot used to determine the protein concentration. The remaining lysate was mixed with 2 × SDS-PAGE loading buffer and heated at 90 °C for 5 minutes.

Proteins were resolved by SDS-PAGE, then transferred to nitrocellulose membranes. Membranes were blocked with 5% non-fat milk and probed with primary antibodies. After washing, IRDye 800CW or 680RD secondary antibodies (1:10,000 dilution, Licor Biosciences, Lincoln, NE) were used to visualize proteins. The membranes were scanned using an Odyssey CLx point-source laser scanner.
**Transfection**

- **Preparation of polyethylenimine (PEI) transfection reagent**

Polyethylenimine (PEI; Polyscience, Warrington, PA) was suspended in water at a concentration of 1 mg/mL and incubated on an orbital shaker until dissolved. The solution was filtered (0.2 µm) and stored at -20 °C until used.

- **Transfection with plasmids**

0.42 µg of plasmid DNA suspended in 50 µL of 150 mM NaCl. 2 µg of polyethylenimine (PEI) was suspended in 50 µL of 150 mM NaCl. The two solutions were mixed and vortexed vigorously for 10 seconds. The reaction mixture was incubated at room temperature for 30 minutes. The reaction mix (100 µL) was seeded as a bolus into a 24 well plate. 50,000 C2C12 cells suspended in 900 µL growth medium were seeded into the well, to make a final volume of 1 mL. The plate was transferred to a tissue culture incubator and incubated for 24 hours prior to analysis or further treatments.

- **Transfection with siRNA constructs**

Stock solutions (20 µM) of siRNAs (Origene, Rockville, MD) were prepared as directed by manufacturer. The reaction mix was formed by adding 30 pmoL siRNA diluted in 150 mM NaCl to PEI diluted in 150 mM NaCl to form complexes of varying DNA/PEI ratios. The siRNA and PEI solutions were mixed and incubated at room temperature for 30 minutes. 20 µL of this reaction mix was aliquoted into a well on a 96 well plate and 180 µL of C2C12 cell suspension (4.4 × 10^4 cells mL^{-1} in GM) was added. After 24 h the cells were washed cells and add fresh medium added. After 48 h the GM was replaced with DM.
Polymerase Chain Reaction

- RNA isolation

Adherent cells were trypsinized, collected by centrifugation, and washed once in PBS, suspending them in a final volume of 100 µL. To this, 1 mL of Tri reagent (Sigma-Aldrich, St. Louis, MO) was added. The tube was shaken for 10 seconds and incubated at room temperature for 5 minutes. Then, 0.2 mL of chloroform (Sigma-Aldrich, St. Louis, MO) was added to each sample, the tubes shaken again, and incubated for 10 minutes at room temperature. The tubes were centrifuged at 12,000 xg for 15 minutes. The aqueous layer (approx. 0.5 mL) was carefully aspirated and transferred to a new tube. To this was added 0.5 mL of 2-propanol (Sigma-Aldrich, St. Louis, MO) as well as 0.1 mL of a 2M NaCl solution. The tubes were incubated at -20°C for 30 minutes, then centrifuged at 12,000 xg for 20 minutes to pellet cellular RNA. The supernatant was aspirated and the pellet washed with acetone. The pellet was allowed to air dry in a fume hood before being resuspended in 50 µL of nuclease-free water. The yield (µg RNA/µL) and quality (260/230 and 260/280 ratios) of each RNA sample was assessed using a NanoDrop spectrophotometer (NanoDrop Products, Wilmington, DE).
- cDNA synthesis and PCR

Total RNA isolates were subject to RT-PCR using a Qiagen OneStep RT-PCR kit (Qiagen Inc., Valencia CA) according to the manufacturer’s instructions. Briefly; reaction mixes were formulated according to the following protocol:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume/reaction</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>5X RT-PCR buffer</td>
<td>10 µL</td>
<td>1X</td>
</tr>
<tr>
<td>dNTP mix</td>
<td>2 µL</td>
<td>400 µM</td>
</tr>
<tr>
<td>Forward Primer</td>
<td>2 µL</td>
<td>0.6 µM</td>
</tr>
<tr>
<td>Reverse Primer</td>
<td>2 µL</td>
<td>0.6 µM</td>
</tr>
<tr>
<td>Enzyme mix (RT &amp; DNA pol)</td>
<td>2 µL</td>
<td>As directed</td>
</tr>
<tr>
<td>RNase inhibitor</td>
<td>1 µL</td>
<td>10 U</td>
</tr>
<tr>
<td>Template RNA</td>
<td>~5 µL</td>
<td>1 µg</td>
</tr>
<tr>
<td>RNase-free water</td>
<td>~26 µL</td>
<td>-</td>
</tr>
</tbody>
</table>
The tubes were transferred to a Veriti Gradient Thermal Cycler (Applied Biosystems, Waltham, MA) and PCR was performed according to the following protocol:

<table>
<thead>
<tr>
<th>Cycles</th>
<th>Stage</th>
<th>Time</th>
<th>Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>1X</td>
<td>Reverse transcription</td>
<td>30m</td>
<td>50°C</td>
</tr>
<tr>
<td>1X</td>
<td>DNA Pol activation</td>
<td>15m</td>
<td>95°C</td>
</tr>
<tr>
<td>45X</td>
<td>Denaturation</td>
<td>1m</td>
<td>94°C</td>
</tr>
<tr>
<td>45X</td>
<td>Annealing</td>
<td>1m</td>
<td>Variable (50-68°C)</td>
</tr>
<tr>
<td>45X</td>
<td>Extension</td>
<td>1m</td>
<td>72°C</td>
</tr>
<tr>
<td>1X</td>
<td>Final extension</td>
<td>10m</td>
<td>72°C</td>
</tr>
</tbody>
</table>

- **Agarose gel electrophoresis**

Samples were mixed with 5X Colourless GoTaq Flexi loading buffer (Promega, Madison, WI) and run on a 2% agarose gel in TBE buffer containing 1:10,000 Sybr Safe (Invitrogen, Waltham, MA). Bands were visualized using a Pharos FX Plus laser gel scanner (Bio-Rad, Hercules, CA).

**Preparation of plasmid DNA**

- **Preparation of competent E. coli cells**

DH5-Alpha E. coli cells (gifted from another lab) were grown in overnight cultures of 50 mL. The cells were chilled on ice, then pelleted for 10 minutes at 10,000 xg. The cells were gently resuspended in cold 0.1M CaCl in H2O and incubated on ice for 20m. The cells were centrifuged again, and then resuspended in cold 0.1M CaCl with 15% glycerol. The cell stocks were aliquoted and frozen at -80°C.
- Transformation of E. coli cells

Glycerol stocks of competent DH5-Alpha E. coli cells were thawed on ice. 100 ng of plasmid DNA was added to 50 µL of cell suspension and mixed gently with the pipette tip. The mixture was incubated on ice for 30m, then briefly transferred to a 42 C water bath for 30s. The cells were returned to ice for 2 minutes, before being inoculated into antibiotic-free LB medium and incubated in a 37 C shaker for 45m. The cells were streaked onto an LB-agar plate containing the appropriate antibiotic:

- Ampicillin 100 µg/mL
- Kanamycin 60 µg/mL
- Chloramphenicol 30 µg/mL

After 24h, colonies were picked and incubated into 5 mL cultures in antibiotic-LB for a further 24h.

- Purification of plasmid DNA

Overnight cultures were subject to DNA extraction and purification using the Qiagen Miniprep Kit (Qiagen Inc, Valencia, CA) according to the manufacturer’s instructions. Briefly; overnight cultures were resuspended, lysed in alkaline buffer, neutralized, and cell debris pelleted. The supernatant was loaded onto a silica column, centrifuged, washed, and plasmid DNA was eluted using EB (10 mM Tris-Cl, pH 8.5). The yield (µg DNA/µL) and quality (260/230 and 260/280 ratios) of each extraction was assessed using a NanoDrop spectrophotometer (NanoDrop Products, Wilmington, DE). The purified DNA was run out on an agarose gel and stained with SYBR Safe to confirm the presence of single bands corresponding to the expected size. Optionally, a
diagnostic restriction digest was performed to further confirm the identity of the plasmid. Reaction mixtures were formulated according to the following:

- 10X reaction buffer: 2 µL
- Restriction enzyme(s): 1 µL (1U each)
- DNA: 1 µg
- Nuclease-Free H2O: 16 µL

The mixtures were incubated at 37°C for 1h, before being run out on an agarose gel and visualized with SYBR Safe.

**Data analysis & statistics**

All numerical datasets with greater than two groups were first tested for normality with D'Agostino's K-squared test. Following this, one-way ANOVA was used to test for statistical significance within the dataset, and Dunnett’s post-hoc test was used to compute mean differences and assess statistical significance. All datasets with two groups were subject to Student’s unpaired t-test. Data analysis was performed using GraphPad Prism version 5 (GraphPad Software Inc, CA). Asterisks indicate significance at <0.05.
General Introduction
General Introduction

The goal of this thesis is to investigate the role of DNA damage in myogenesis. This introduction will give background material of each of the relevant fields, and is divided into three sections. *Muscles & myogenesis* will describe the structure of skeletal muscle and the process of myogenic regeneration. *Cell death & caspases* will describe the phenomenon of apoptotic cell death and its effector proteases, the caspases, as well as their relation to the process of myogenic regeneration. *DNA damage & differentiation* will cover different types of DNA damage, as well as the DNA damage response, and will outline evidence for a role for DNA damage in differentiation.

Muscles & myogenesis

_Gross anatomy of skeletal muscle_

Muscle is a specialized form of soft tissue characterized by its ability to produce percussive contractions. This is the result of the action of individual actin-myosin elements within each muscle fiber. These elements generate mechanical force when stimulated by the nervous system. There are several different kinds of muscle, including skeletal muscle, cardiac muscle, and smooth muscle. Skeletal muscle is under the control of the somatic nervous system, and its activity can be controlled voluntarily. Cardiac and smooth muscle are non-voluntary and are controlled by the autonomic nervous system.
Skeletal muscle is a major tissue type in humans, comprising approximately 40% of total body mass in adults (Marieb & Hoehn, 2010). Skeletal muscle gives structure and motility to the body, and allows for voluntary locomotion as well as involuntary postural reflexes. Skeletal muscle is anchored to the skeletal system through fibrous ligaments which allow a measure of elasticity between the fiber bundle and the bone. Individual skeletal muscle bodies are structured with several nested layers of organization (Diagram 1).

**Diagram 1: Structural overview of skeletal muscle.** The muscle body is attached to the bone through a collagenous ligament. The muscle body is wrapped in a thick layer of connective tissue known is the epimysium. Each muscle body is composed of nested bundles of fascicles, which themselves contain bundles of myofibers. Myofibers contain the functional actin-myosin elements which allow for muscle contraction. Regenerative satellite cells are studded along the outer surface of each myofiber.
Each muscle bundle is enveloped in a thick layer of fibrous tissue known as the epimysium, and is attached to the bone through a collagenous ligament. The epimysium encloses a number of muscle fascicles, surrounded by perimysium. The perimysium also contains blood vessels to provide oxygen and allow access to immune cells. Each muscle fascicle in turn contains individual myofibers, which are elongated, multinucleated syncytia. *In utero*, myofibers are formed through the fusion of individual myocytes. As the myofibers develop, the cytoplasm is gradually dominated individual actin-myosin filaments, organized into sarcomeres. These are the functional units of muscle contraction. The nuclei of each myofiber are moved toward the outer surface of the cell body to make room for sarcomeres. Along the outer surface of each elongated myofiber, known as the basal lamina, are neuromuscular junctions which innervate the fiber and place it under the control of the somatic nervous system. The basal lamina is also home to the satellite cells. These are a population of quiescent cells which are activated in response to muscle injury and provide a regenerative reservoir to allow for repair.
Muscle regeneration

Injuries to the skeletal muscle can occur as the result of a number of factors, including blunt trauma and lacerations, physical exercise, and drug use. Muscle injuries often manifest as tears of individual myofibers. Upon injury, a process of repair and regeneration is initiated (Diagram 2).

**Diagram 2: Myofiber repair on the macroscopic level.** Each end of the ruptured fiber exudes a process into the interstitial space. These processes connect, and tether the two fiber segments together. At the site of the tether, a dense cluster of nuclei and cell aggregate is formed, which gradually develops into a mature myofiber segment. The contractile apparatus is eventually restored and functionality returns.

On a macroscopic level, each ruptured fiber exhibits a wave of cell death at the periphery of the site of injury. From each end of the torn fiber, a process is extruded towards the partner fiber. These processes make contact and undergo anastomotic fusion, reconnecting the torn fiber ends. This connection develops into a ‘bulge’ which is rich in nuclei arranged in a disordered fashion.
Gradually, the fiber is repaired and takes on a thicker appearance at the former site of the rupture (Gay & Hunt, 1954). This new connection has a stronger tensile strength than the previous segment of fiber, giving the fiber a higher resistance to future insults. This fiber strengthening is the basis of muscle hypertrophy, which occurs as the result of physical exercise and weight training (Petrella, 1985).

In mammals, this process of myofiber repair is imperfect, as it can lead to the formation of scar tissue and denervation which can permanently impair fiber function. In other higher animals such as salamanders, axolotls, and other members of the Caudata clade, myofiber regeneration is much more sophisticated, involving a process of coordinated myofiber dedifferentiation into individual myocytes, followed by their re-fusion into new fibers. This is a time- and energy-expensive process, however, and mammals have opted for the ‘quick and dirty’ approach of directly ligating ruptured myofiber ends together.
Myogenic satellite cells

On the cellular level, muscle fiber regeneration is the responsibility of satellite cells. Satellite cells are quiescent cells which are found embedded along the basal lamina on the outside surface of myofibers. Satellite cells as a distinct population found adherent to isolated muscle fibers were first reported in 1961 (Mauro), and by 1975 they had been demonstrated to be the primary substrate of myogenic fusion (Bischoff; Konigsberg). When a myofiber lesion occurs, these cells are awoken from their quiescent state, and begin to proliferate and express myogenic commitment factors such as MyoD, and to lose stem cell markers such as Pax7 (Zammit, 2006; Le Grand & Rudnicki, 2007). This marks their transition from satellite cells to committed myoblasts.

As they migrate to the site of injury, these daughter cells begin to fuse into myosin-rich nascent myofiber segments. These segments then undergo secondary fusion to the site of injury, bridging the disjunction (Diagram 3). Historically, there was controversy about whether myofibers come about as the result of cell fusion, or as the result of some form of crypto-mitosis, in which the nuclear DNA is duplicated, but cytokinesis is interrupted, resulting in a syncytium. Sequentially repeating this process would result in the formation of multinucleated myocytes resembling myofibers. Experiments in the 1960s demonstrated that in vitro myotube development can be induced even when DNA replication is blocked (Konigsberg, 1960). This seemingly precluded the possibility of such a nuclear replication mechanism. Modern fluorescent videomicroscopy techniques allow the process of myoblast fusion to be viewed from start to finish (Millay, 2013).
Diagram 3: Process of satellite cell activation and fusion in muscle regeneration. After the muscle fiber has sustained a break, a large number of dead and dying cells are present at the site of the break. Cell death also occurs within the residual fiber segments distal to the site of injury, presumably in a pruning process to remove ragged necrotic fiber ends and create a ‘clean’ break. There is generally also inflammatory infiltrate into the break site in order to remove dying cells and debris. In response to fiber breakage, quiescent satellite cells along the basal lamina of the myofiber become activated. These activated satellite cells are attracted to the site of damage, and undergo proliferation. The daughter cells then fuse into individual myofiber segments, which themselves undergo secondary fusion to bridge the gap between the myofibers. This stage corresponds to the aggregate of cell mass and nuclei seen in the macroscopic view of fiber regimentation described in Diagram 2 above.
The fact that activated satellite cells are attracted to the site of injury suggests that some chemotactic factor emanates from the damaged milieu, in essence creating a line of communication between the site of injury and the niche within which satellite cells reside. After a myofiber break, the site of damage is rich in dying cells, both apoptotic and necrotic. It is conceivable that these dying cells are the source of this chemotactic signal.

Terminal myogenic differentiation involves the activity of a coordinated program of transcription factors which direct the cell fate towards differentiation. Principal among these transcription factors are the Myogenic Regulatory Factors (MRFs), which include MyoD, myogenin, Myf5, and MRF4. Each of these transcription factors initiates a cascade of further transcriptional activation and repression, controlling the expression of hundreds of different transcripts (Moran, 2002; Kislinger, 2005). These gene expression changes control the visible morphological features of the differentiation program, such as enabling chemotactic motility, engaging the synthesis of myosin and other muscle-associated structural polymers, and withdrawal from the cell cycle into a postmitotic state. These gene expression changes ultimately result in the fusion of myoblasts into multinucleate myofibers. This process can be simulated in vitro using immortalized cell lines, and this practice forms the basis for the experimental work presented in this thesis. Myoblastic cell lines have been available for many years, and have yielded advances in understanding the process of muscle development that have translated well into in vivo settings (Shoji, 2015). The goal of this thesis is to investigate the overlap between the mechanisms of myogenic regeneration and apoptotic cell death. In the next section, an overview will be presented of the apoptotic cell death field and the current understanding of the role of apoptotic mechanisms in myogenic differentiation.
Cell death & caspases

Cell death

Cell death can occur under a number of circumstances. Cells may die when they are exposed to genotoxic or cytotoxic conditions, cells may die when they are infected with microbes or other pathogens, or they may die simply when they reach senescence and can no longer proliferate. There are several well established and distinct modes of cell death that appear to represent the major mechanisms of cell death in living organisms.

In general, the broadest distinction is made between regulated and unregulated forms of cell death. The major point of distinction is that regulated forms of cell death proceed through controlled biochemical and metabolic pathways in a step-wise fashion. In theory, a regulated form of cell death can be aborted if any of these sequential steps is blocked. Unregulated cell death does not feature such control mechanisms. The most well-known form of unregulated cell death is necrosis, a broadly-defined term encompassing non-programmed cell death where the cell is physically destroyed or rendered non-viable as the result of sheer physical or chemical trauma. The most studied form of regulated cell death is apoptosis, a highly coordinated form of programmed cell death. Necroptosis is a process which lies between regulated and unregulated forms of cell death, as it appears to be controlled by a much smaller program comprising 2-3 kinases (the RIP kinases and MLKL). Necroptosis has been described as a ‘back-up’ or ‘trapdoor’ process which is initiated when apoptotic cell death is prevented from occurring.

It is important to note that cell death is not solely a destructive process. Early cell biologists recognized that cell death is essential for maintaining tissue homeostasis, acting as a counter-
balance to cell proliferation in order to maintain the structure and functionality of tissues and organs (Zakeri, 2008; Clarke, 1996). Along with this, it has been demonstrated that dying cells release compounds which act as paracrine signaling factors to influence the behavior of other cells. For example, virus-infected cells which are undergoing cell death may shed chemotactic factors in order to attract cells of the immune system (Klimpel, 1996). Another example may be cells which are dying as a result of tissue trauma releasing signaling factors to activate nearby stem cells in order to kickstart the regenerative process (Li, 2010).

**Apoptosis**

Perhaps the most well studied mode of regulated cell death to date is apoptosis. A form of cell death which is distinct from cell necrosis was first described in 1965 (Kerr). In this mode of cell death, cells remained physically intact as they died, and these intact cells were resorbed by histocytes. By 1972, this had proposed as a mode of programmed cell death termed 'apoptosis' (Kerr).

The most distinguishing feature of apoptosis is the morphological changes that occur (reviewed in Hacker, 2000). Apoptotic cells shrink significantly in volume, while the cell membrane takes on a convoluted, raspberry-like topology, with distinctive ‘blebs’ developing on the surface. These blebs are later shed off as apoptotic bodies as the cell disintegrates. During this process, the apoptotic cell typically loses its anchorage to neighboring cells and becomes free floating. Within the cell, the nucleus condenses in size, and the chromatin is broken up into nucleosomal particles. Outside the nucleus, the cytoskeleton and other structural features are systematically dismantled by proteases. These genomic fragments, structural elements, organelles and mitochondria are
enclosed within apoptotic bodies, which finally lose cohesion and are dispersed as separate intact lipid-bound bodies. These bodies are typically taken up by other cells and degraded by autolytic processes.

In terms of signaling, apoptosis can be divided into at least three distinct pathways. These are the extrinsic, intrinsic, and immunogenic pathways. All three pathways are triggered by distinct signal events, and all three ultimately converge on the activation of executioner caspases, the main functional effectors of many of the morphological events outlined above.

The extrinsic apoptotic pathway (reviewed in Wallach, 2008) is triggered by binding of death ligands to death receptors on the plasma membrane (Diagram 4). Among these death receptors are the TNF receptors, the Fas receptor, the TRAIL receptors, and the TWEAK receptor. There are also quite a number of ‘decoy’ receptors which can bind ligands but not transmit intracellular signals, used by cells to regulate the efficacy of death ligand signaling. These receptors are members of the TNF receptor family, named for the first identified ligand, all share structural homology (Naismith & Sprang, 1998). Each death receptor harbours a cytoplasmic domain referred to a ‘death domain’. Upon ligand binding, death receptors oligomerize and form the DISC Death-Inducing Signaling Complex (DISC) (Kischkel, 1995). Adaptor molecules such as FADD and DEDD are activated through this mechanism, and in turn activate upstream caspase such as caspase-8 and -10 (Alcivar, 2003). These upstream caspases have proteolytic activity, however their primary function is the activation of executioner caspases. Once activated, the executioner caspases-3 and-7 cleave substrates necessary to affect cell shrinkage and membrane blebbing (including actin, GAS-2, α-fodrin, and ROCK-1, as well as substrates involved in DNA
fragmentation and chromatin condensation (such as CAD and acinus) (Fischer, 2003). Death ligands are often generated by immune cells in order to trigger apoptosis in viral-infected or otherwise abnormal cells (Waring, 1999). It is important to note that extrinsic death signaling is often manipulated by cancer cells in order to circumvent cell death and allow unrestricted proliferation (Fulda, 2010) (although it is also the case that intrinsic death signaling can be manipulated in cancers, for example through upregulation of anti-apoptotic Bcl proteins or IAPs).
Diagram 4: A simplified overview of the extrinsic and intrinsic apoptotic pathways. The extrinsic pathway is initiated by death receptor signaling, and leads to caspase-8 activation. The intrinsic pathway is centered on mitochondrial membrane depolarization, and leads to caspase-9 activation via the apoptosome. Both pathways ultimately converge on caspase-3 activation.
The intrinsic apoptotic pathway is typically activated as a response to toxins, hypoxia, radiation, or DNA damage (reviewed in Estaquiet, 2012). In general, the extrinsic pathway is engaged as a consequence of direct toxicity or insults within the cell, as opposed to the signal-driven activation of the extrinsic pathway. Through different self-sensing mechanisms, this cellular damage leads to the activation of pro-apoptotic members of the Bcl-2 family (Hockenberry, 1990). These proteins are thought to interfere with the activity of anti-apoptotic Bcl-2 proteins on the mitochondrial membrane, which ultimately leads to the opening of anion channels, disrupting the electrical potential of the mitochondrial membrane and allowing the release of cytochrome C (Oltvai, 1993). Once in the cytoplasm, cytochrome C associates with Apaf-1 and caspase-9 to form the apoptosome, a large heptameric structure which acts as a platform from which caspase-9 can activate executioner caspases (Yuan, 2013). Although they are treated as conceptually separate, there can often be cross-talk between the intrinsic and extrinsic (Roya, 2000). For example, the extrinsic pathway can, through BID, lead to the activation of the mitochondrial pathway (Sprick, 2004). Additionally, there is evidence that executioner caspase-3 can induce a retrograde activation of both caspase-8 (Ferreira, 2008), which may make it even more difficult to tease apart the two pathways in experimental systems.

The third mechanism of apoptosis induction is the immunogenic pathway (Diagram 4). This pathway is initiated by the transfer of granzyme B and perforins from cytotoxic T lymphocytes to target cells. The perforins mediate entry into the cell of granzyme B. Granzyme B is a serine protease with a large number of cellular substrates, and can induce apoptosis by several different methods (Chinnaiyan, 1996). It can cleave initiator caspases-8 and -10 to their active forms, it can directly activate caspases-3 and -7, and it can induce mitochondrial membrane depolarization
Granzyme B can also halt DNA copying and repair through cleavage of Topoisomerase 1, PARP, and DNA-PK (Casciola-Rosen, 1999). As well as this, granzyme B can induce anoikis through cleavage of several extracellular matrix proteins including vitronectin and fibronectin (Buzza, 2005).

Altogether, the immunogenic pathway can induce many of the features of apoptosis, either through commandeering the cells own apoptotic pathways, or simply through direct cleavage of apoptotic substrates. As opposed to the intrinsic apoptotic pathway, in which the cell activates its own proteolytic cascase leading to death as a result of internal self-sensing mechanisms, the immunogenic pathway seems to represent a direct killing of the cell by immune effector cells (murder rather than suicide). Since this process is not induced in an autonomous fashion, nor does it appear to be regulated at the cellular level, the immunogenic pathway is sometime not considered a genuine form of apoptosis; however the biochemical and morphological features of this form of cell death are certainly reminiscent of those of the canonical pathways.

**Caspases**

Caspases are a class of cysteine-aspartic acid endoproteases. Other members of the cysteine peptidase family include the cathepsins, the calpains, papain, and cruzipain. Caspases are primarily found in higher animals, although orthologous enzymes are found in plants, insects, and molds (Koonin, 2002). These enzymes are considered to be primarily involved in programmed cell death. Many of these cysteine proteases share a catalytic triad involving an initial deprotonation of the substrate thiol group, formation of a thioester intermediate, which is finally hydrolyzed into a carboxylic acid moiety. Caspases themselves recognize a motif of four or more residues which is
schematically represented as P4-P3-P2-P1. Cleavage occurs at the C-terminal of P1, which is typically an aspartic acid residue (McStay, 2008). The substrate-binding groove is nearly identical for all caspases which have been studied.

Depending on how they are classified, there are as many as twelve caspases currently identified in humans (Chowdhury, 2008). Caspases are typically divided into three classes with respect to their role in apoptosis; initiator caspases, effector ("killer") caspases, and non-apoptotic caspases. Initiator caspases include -2, -6, -8, -9, -10, and -15. They are distinguished from effector caspases by prodomain interaction motifs like CARD and DED (Kumar, 1999). Their primary role in apoptosis is the proteolytic activation of effector caspases. Initiator caspases are thought to self-activate by induced proximity or conformation change to form active dimers. Effector caspases include -3, -6, and -7 (Cohen, 1997; Elmore, 2007). The main functional difference between initiator and executioner caspases appears to be related to either substrate specificity or substrate accessibility during the period of their activation, with initiator caspases having a much more limited set of available substrates as compared with the downstream caspases, for which hundreds or thousands of substrates exist.

It is important to note that these designations reflect only the first identified function of each protease, and not necessarily its only function. Indeed, most caspases been found to have roles unrelated to apoptosis, as detailed in the next section.
Kinder killers? Executioner caspases in non-apoptotic roles

Caspases were originally discovered in the context of inflammation research (Thornberry, 1992), although most of the early research concerning caspases was in the context of their role in programmed cell death (Ellis, 1986; Yuan, 1993). This was especially the case after mammalian homologs of Interleukin-1β Converting Enzyme (ICE; caspase-1) were discovered (Nicholson, 1995) and their potential applications in medical research and treatment were realized.

Given this precedent, it has generally been assumed that the downstream “killer” caspases have roles only in cell death. This is not an unreasonable assumption. Initiator caspases serve primarily to activate executioner caspases, and executioner caspases have several characteristics that would seem to preclude them from having non-apoptotic roles. First, the cleavage motif: caspases recognize short, four residue motifs, typically including an aspartic acid. Because this recognition motif is so small, it is very common. The number of currently-identified caspase targets is approaching eight hundred (Lüthi, 2007). Since many of these have been identified through systematic cell extract screening, the corpus of known substrates probably represents a large fraction of all existing caspase substrates, at least in mice and humans.

All things being equal, a single active caspase enzyme within the cell should be able to cleave thousands of proteins. And it gets worse. Caspases also have the ability to autoactivate. That is, an activated initiator caspase can activate other initiator caspases by cleaving their zymogen form. In theory, a single activated inititator capsase within a cell could unleash a chain reaction of initiator caspases, followed by effector caspases, leading to an irreversible proteolytic cascade which
degrades every major structure in the cell. In light of this, it seems impossible that active caspases can be tolerated by the cell. And yet they are.

The first non-apoptotic roles found for caspases did not come as a conceptual shock, because these roles all morphologically resemble apoptotic cell death. In 1998, it was discovered that the enucleation of epithelial lens cells relied on caspase activity (Ishizaki, 1998). Likewise, Zermati (2001) reported that enucleation of erythrocytes was also dependent upon caspases, and Weil (1999) showed that the same was true for keratinocyte enucleation. Although novel roles for caspases, each of these could be explained as a form of ‘interrupted apoptosis’, where a canonical apoptotic pathway has been unleashed, but then blocked by some mechanism or other. The chromatin was fragmented, the nucleus was condensed and eventually lost, but other signs of apoptosis were absent. The next decade brought more surprises, however.

De Botton (2002) showed that megakaryocytes, which give rise to platelets, require localized caspase activity. This happened in the absence of DNA fragmentation, and seemingly also in the absence of other markers of apoptosis. Likewise, Fernando (2005) determined that caspase activity was required for neuron development, and it was later found that this involves discrete, localized caspase activity within the cell body. Sordet (2002) reported a role for caspases in monocyte differentiation, seemingly in the absence of any apoptotic-like phenotype at all; and the same was found to be true for Bergmann glial cells (Oomman, 2006). Caspases were also implicated in germ-line cell development (Arama, 2003), as well as in true stem cells (embryonic stem cells, hematopoietic stem cells, and placental trophoblast cells) (Fujita, 2008; Janzen, 2008; and Black, 2004 respectively). Of most interest to this thesis, the intrinsic apoptotic pathway was implicated
in the fusion of myoblast cells into mature myotubes. This requires both caspase-3 (Fernando, 2002) and caspase-9 (Murray, 2008).

Caspases have now been implicated in many cellular processes, not just cell differentiation. These processes often involve large-scale modifications to the cell as a whole, such as migration and motility, cytoskeleton rearrangement, projection of cytoplasmic membrane elements, and the fusion of cell membranes (reviewed in Connolly, 2014).

Caspases in muscle differentiation: state of the field

The role of caspases in myogenic differentiation was originally demonstrated in 2002 (Fernando, 2002). Since then, several more key reports have been published and two schools of thought have emerged to interpret the growing body of literature regarding caspase activity in myogenic differentiation.

The first school of thought is more conservative. According to this interpretation, caspase activity is localized strictly within apoptotic cells. When myoblasts are induced to differentiate, a sizable fraction (perhaps one third) of the cells undergo apoptotic cell death. The apoptotic interpretation is that these dying cells shed signaling factors which kickstart the differentiation programs of the survivors. This is in line with a growing body of literature concerning compensatory proliferation and apoptosis-induced cell signaling (Li, 2010). Apoptotic cells are not dead, inert bodies; they are more like ghosts, still present in the cellular milieu and able to exert influence on other cells through signaling factors, until they are put to rest by immune cells.
The key evidence for the apoptotic signaling interpretation is an experiment conducted by Hochreiter-Hufford (2013). In this experiment, supplementation of myoblasts with pre-generated apoptotic myoblasts induced them to differentiate spontaneously, even in the presence of caspase inhibition. This is taken to indicate that cell-cell contact from apoptotic cells is sufficient to induce differentiation (Although this could also be due soluble factors shed from the apoptotic cells, since they are not chemically fixed, and could continue to secrete components into the culture medium). An issue with this experiment is that this apoptotic cell-cell contact is mediated by phosphatidylserine exposure on the apoptotic cell surface, and its recognition by the BAII receptor on the corresponding myoblast. An issue with this interpretation is that healthy myoblasts also expose phosphatidylserine as part of their differentiation program. Myoblasts present phosphatidylserine on their cell surface, at the point of cell-cell contact just prior to fusion (van den Eijnde, 2001). This membrane inversion itself may be a caspase-induced event, just as it is in apoptosis. It is possible that in the Hochreiter-Hufford experiment, the apoptotic myoblasts are simply mimicking a true non-apoptotic caspase-dependent event.

The other school of interpretation posits a truly non-apoptotic, cell-autonomous role for caspases. In this interpretation, the proteolytic activity of caspases are restrained and harnessed for other cellular processes, quite distinct from apoptosis. Caspases are seen as having a wider range of roles than previously imagined, all involving large-scale reorganization or cell fate changes. Caspases can be thought of a self-modification enzymes which enable the cell to carry out these changes.

True non-apoptotic roles for caspases have been discovered in other systems, including spermatid development (Arama, 2002). In *Drosophila*, sperm are created in cystic bulges of 64 connected
spermatids. The process of individualization involves the shedding of bulk cytoplasm, which disconnects each spermatid from its neighbor. The caspase-3 ortholog Drice is required for this gathering and shedding of the cytoplasm. Drice activity proceeds in a wave down the length of the spermatid. Drice activity relies on cytochrome C, suggesting an apoptotic-like activation mechanism. Interestingly, it was shown that Drice activity is spatially restricted by the IAP-like ubiquitin ligase dBruce in order to prevent nuclear degradation in the head region. This represents a mechanism through which caspase activity can be harnessed and controlled in order to be used for non-apoptotic applications.

The evidence for the non-apoptotic interpretation of caspase activity in skeletal muscle comes from histological and tissue culture studies. Histological studies of regenerating muscle fibers show cells which stain positive for caspase activity and DNA strand breaks (Tews & Goebel, 1997; Ikeda, 2009; Coulton, 1992). These cells do not have a gross apoptotic morphology and appear to be otherwise normal. Given the location of these cells within the basal lamina (the home of quiescent satellite cells), as well as central within the regenerating fiber (where the nuclei of newly-attached differentiated satellite cells are located), it is inferred that the caspase-positive cells are the myogenic precursors (Sudo, 2009). It has independently been shown through in vitro experiments that myoblasts accumulate DNA damage as they differentiate (Farzaneh, 1982), which is later repaired (Dawson & Lough, 1988) and that caspase activity is required for myoblast differentiation (Fernando, 2002), as well as for producing myogenic DNA damage (Larsen, 2010). Together, this has been taken to present a narrative in which a cell-autonomous caspase activity is used to generate DNA strand breaks, and that these lesions precede and are causal of myogenic gene transcription.
To date, the definitive experiment to test whether or not myogenic caspase activity is cell autonomous has not been conducted. Such an experiment would involve lineage-tracking of myoblasts with a fluorescent marker for caspase activity. If a cell were to be observed unleashing caspase activity, while remaining morphologically non-apoptotic, and if that cell were observed to undergo fusion to a nascent myotube, this would constitute convincing evidence that caspase activity could be sustained within a living cell as part of its terminal differentiation program.
DNA damage & differentiation

DNA damage

The goal of this thesis is to investigate the phenomenon of DNA damage during myoblast differentiation. DNA damage can be broadly defined as any change in the structure of a DNA strand. This encompasses a very wide range of different kinds of events. These include chemical modifications of individual nucleobases such as oxidation, alkylation, depurination, and depyrimidination; as well as alterations in the structure of the strand as a whole, such as adduct formation, strand crosslinking, chemical intercalations, and pyrimidine dimer formation. Other types of DNA damage include substitution of mismatched nucleobases, i.e. those which do not fit the A-T and G-C pairing rules. Each of these structural alterations can lead to DNA strand breaks. Strand breaks represent a major category of DNA damage. These are divided into two classes; single-stranded and double-stranded DNA breaks. Single-stranded breaks are formed through loss, mismatch, or chemical damage to bases on a single strand of DNA. Double-stranded breaks occur when damage is sustained to complementary bases on both strands, leading to their physical separation.

DNA damage can occur as the result of exogenous or endogenous sources. Exogenous sources of DNA damage include sunlight and other sources of radiation, integrating viruses and other pathogens, environmental chemicals and pollutants, medications and street drugs, as well as various foods, alcohol, and tobacco. Endogenous sources of DNA damage include products of
metabolism (particularly reactive oxygen species), errors in DNA replication, and deliberate strand breakage through the activity of endogenous nucleases. It has been estimated that each mammalian somatic cell accumulates as many as 50,000 single stranded breaks and 50 double stranded breaks per day (Villenchik & Knudson, 2003; Tice & Setlow, 1985). As such, DNA damage represents a constant threat to the cell, and the genome must be continuously monitored and repaired.

The DNA damage response

Mechanisms have evolved which allow the cell to efficiently detect and repair DNA damage. These mechanisms are collectively called the ‘DNA damage response’. The DNA damage response can be conceived as a multilayer hierarchy of sensors, transducers, and effectors (Diagram 5). Sensors directly bind to the site of damage and different sensors bind to different kinds of DNA damage. In mammals, the major DNA damage sensors include ATM, the ATR/RPA complex, and the DNA-PK/Ku70/Ku80 complex. Sensors can bind DNA lesions with remarkable efficiency. One recent publication showed that binding of Ku is detectable less than 5 minutes after laser micro-irradiation of the nuclear chromatin (Britton, 2013).

DNA damage sensors activate signal transducers. These are intermediary proteins which mobilize downstream components of the DNA damage response. One such signal transducer is histone 2AX (H2AX), which in its phosphorylated state recruits factors such as NBS1, 53BP1 and BRCA1 (Ward, 2003; Kobayashi, 2002; Paull, 2000). Signal transducers can operate in multi-enzyme cascades through several intermediaries. The function of these proteins is to recruit and coordinate the downstream effectors of the DNA damage response.
Diagram 5: Schematic overview of the DNA damage response. DNA lesions are directly bound by sensor molecules such as the ATM, ATR, or DNA-PK complexes. These sensors activate signal transducers, which often involve a multistep cascade of enzyme interactions. The signal transducers activate effectors, which implement cell fate choices. The system as a whole determines the appropriate cell fate choice, for example the type of DNA repair pathway to be activated.
In response to DNA damage, the cell must decide between several fate choices (reviewed in Zhou & Elledge, 2000). The cell can halt the cell cycle and attempt to repair the DNA lesion, or it can alter gene transcription, or it can undergo programmed cell death. The downstream effectors of the DNA damage response are responsible for carrying out these cell fate choices. How the cell decides between these choices, particularly between the choices of DNA repair of programmed cell death, is still incompletely understood, although the choice certainly depends upon the extent of the DNA damage incurred (Tian, 2012).

**DNA repair pathways**

Different forms of DNA damage activate different DNA repair pathways. These generally consist of macromolecular complexes of proteins which bind at the site of DNA damage, excise or repair any damaged bases, and ligate strand breaks. The major DNA damage response mechanisms activated in response to single-stranded DNA damage are base excision repair, nucleotide excision repair, and mismatch repair.

In brief; base excision repair involves the removal of a damaged nitrogenous base, opening up of the DNA backbone, the removal of the damaged region through the exonuclease activity of DNA polymerase, and the synthesis of the correct complementary strand sequence (Lindahl, 1974; Krokan & Bjørås, 2013).

Nucleotide excision repair is used to repair larger, helix-distorting lesions such as pyrimidine dimers. A nuclease complex removes an entire region of DNA upstream and downstream of the lesion, creating a 12-24 base tract of single-stranded DNA. The complementary strand is then
synthesized by DNA polymerase (Setlow & Carrier, 1964; Boyce & Howard-Flanders, 1964; Pettijohn & Hanawalt, 1964; Shuck, 2008).

Mismatch repair is used to correct mistakes in DNA replication which lead to complementary bases which do not follow the A-T and G-C pairing rules. MutSα and MutLα recognize the incorrect base. A nick incision is created upstream of the mismatch site, and helicase is used to unwind the DNA strands. An exonuclease removes this 'tail', revealing a tract of single-stranded DNA. Polymerase then re-synthesizes the complementary strand (Lu, 1983)

Each of these repair mechanisms relies on the generation of tracts of single-stranded DNA, and the sequence on the complementary, undamaged strand is used as a template for the re-synthesis of the damaged strand.

There are also distinct DNA damage response pathways which are evoked in response to double-stranded breaks. In response to double-stranded DNA breaks, there are also three major pathways; homologous recombination, microhomology-mediated end-joining, and non-homologous end-joining (originally called ‘indiscriminate recombination’). The latter modality is of interest because it does not rely on any template or homology for DNA repair, instead blunt ends are directly ligated.

Homologous recombination is different to the other two double-stranded break repair pathways because it takes advantage of the existance of homolgous sequences on sister chromatids during the late S and G2 phases of the cell cycle. Free DNA ends are bound by the MRN complex. One
strand of each broken end is trimmed by Sae2 and Exo1 to reveal a single-stranded overhang. These overhangs are bound by RPA. Rad51 then searches for a homologous sequence on the sister chromatid, which is then unwound and bound by the ‘invading’ overhang. The undamaged sequence from the sister chromatid is then copied and used to create a repaired strand (Tatum & Lederberg, 1947; Capecchi, 1989; Krejci, 2012).

Microhomology-mediated end-joining takes advantage of overhanging sequences base sequences on the broken DNA ends. Exo1 degrades one strand of each broken end until 5-25 base homologous sequences are found. The homologous sequences are aligned and ligated. Overhanging ‘flaps’ are removed, and missing and mismatched bases are repaired. Microhomology-mediated end-joining is inherently error-prone, because this mechanism of overhang alignment results in deletion of sequences (Nussenzweig, 2007; McVey, 2008).

In non-homologous end-joining, free DNA ends are bound and kept in close proximity by the ku70/80/DNA-PKcs complex. Nuclease activity is used to process the ends to ensure they have compatible 3’ hydroxyl and 5’ phosphate termini, and overhangs are filled in using DNA polymerases. Once the termini are processed, the ends are ligated together, primarily through the activity of DNA ligase IV (Botchan, 1980; Winocour & Keshet, 1980; Wilson, 1982; Davis & Chen, 2013).

It is important to note that within a given cell, many different kinds of lesions are present at a given time. Consequentially, several different DNA damage response modalities are likely to be active concurrently. This is likely also the case when cells are exposed to genotoxic conditions such as
drugs and irradiation. There may be one predominant form of DNA repair present in response to a given genotoxic insult, but other DNA repair pathways can be active at the same time. Thus the DNA damage response may be a mosaic of the different repair pathways outlined above.

**DNA damage as a consequence of caspase activity**

One of the major morphological features of apoptosis is chromatin condensation and fragmentation. This is mostly carried out through the activity of CAD (caspase-activated DNase; also called DNA Fragmentation Factor) (Liu, 1998). CAD is transcribed as a single peptide conjoined to its inhibitor, ICAD (Enari, 1998). During apoptotic cell death, caspase-3 cleaves ICAD, releasing CAD (Wolf, 1999). Other proteins also contribute to apoptotic DNA damage in parallel, including Apoptosis Inducing Factor (AIF) (Susin, 1999) and DNase gamma (Shiokawa, 1994), although mice which lack the DFF45 gene lack both DNA fragmentation and condensation (Zhang, 1998), suggesting that CAD is the primary effector of apoptotic DNA degradation.

During apoptosis, DNA degradation occurs in two stages. In the first stage, the caspase-independent endonucleases AIF and DNase gamma enter the nucleus and cleave the transcriptionally-inactive peripheral chromatin into 50-300 basepair fragments (Joza, 2001; Li, 2001; Susin, 2000). Peripheral chromatin is used to anchor the genomic DNA to the nuclear lamina (Kalverda, 2008), so cleavage of peripheral chromatin presumably releases the genomic DNA from its anchorage and facilitates further degradation. In stage II DNA degradation, caspase-dependent activation of CAD leads to its translocation to the nucleus (Enari, 1998). Once inside the nucleus, CAD cleaves nuclear chromatin into internucleosomal segments of approximately 180 basepairs (as histone-wrapped DNA is inaccessible to CAD) (Wyllie, 1980). This DNA cleavage is a
relatively late event in apoptotic cell death. Apoptotic cells still retain a functional DNA damage response until the terminal stages of the apoptotic program (Mukherjee, 2006), although they do not repair their DNA.

**DNA damage in myogenesis**

It was first shown by Farzaneh (1982) that differentiating culture of myoblasts accumulate DNA strand breaks. This was confirmed *in vivo* by Coulton (1992), who showed that strand breaks occur within regenerating muscle fibers in mice, as well as by Sandri (1995) in humans. Sudo (2009) showed that these strand breaks were localized to nuclei within the sarcolemma and centrally within the regenerating fiber, two locations that are strongly associated with myoblastic satellite cells, suggesting that these cells are regenerative cells.

Larsen (2010) showed that the DNA strand breaks which appear in differentiating myoblast cultures are produced by caspase-activated DNase (CAD). CAD is activated by caspase-3, which itself is active during myogenic differentiation (Fernando, 2002). Inhibition of CAD by siRNA blocks the accumulation of strand breaks, and also blocks myogenic differentiation, suggesting that the activity of CAD is essential for the differentiation program.

Along with DNA damage, DNA repair processes are active during myogenic differentiation. Farzaneh (1985) and Dawson & Lough (1988) demonstrated that DNA strand breaks during myogenic differentiation are transient in nature. Jost & Jost (1994) showed that conventional DNA repair pathways are active during differentiation.
The nature of this myogenic DNA damage has not yet been determined. Firstly, it is not known whether CAD induced DNA damage is present within every cell in the population, or just a sub-fraction of cells. Linked to this, it is also not known whether myogenic DNA damage is localized within actively differentiating cells, or within the sub-fraction of myoblasts which undergo apoptotic cell death when induced to differentiate, although the fact that DNA repair mechanisms are engaged during differentiation suggests that apoptotic cells are not the source of the detected DNA damage, as apoptotic cells do not repair their DNA. Secondly, the distribution of DNA strand breaks within the genome is not yet known. Are these strand breaks produced randomly, or are they targeted to particular sequences? Thirdly, what is the function of this DNA damage? DNA strand breaks are extremely dangerous for the cell, as they can give rise to mutations and chromosomal breaks and translocations. Why do cells deliberately cleave their own DNA, only to immediately repair it? There must be an important downstream function of such risky behavior.

The goal of this thesis is to address all three of these questions.

Work presented in this thesis

This thesis is divided into several chapters which represent distinct but linked bodies of work. In the first chapter I recapitulate several key experiments from the literature with respect to the roles of caspases and DNA damage in myogenic differentiation. I then show that contrary to the current position in the field, DNA damage which is produced by an undirected, non sequence specific method (UV irradiation) is sufficient to drive myogenic differentiation.

In the second chapter, I attempt to identify the nature of the DNA damage response elicited during myogenic differentiation. I show that the key non-homologous end-joining enzyme DNA-PKcs is
required during myogenic differentiation, and is in part responsible for the induction of the myogenic DNA damage response.

In chapter 3, I conduct a screen of a chemical library in an attempt to uncover novel kinases involved in myogenic differentiation. This yielded six hit compounds. One hit, PD-407824, was further investigated because its purported target, Wee1, is a checkpoint kinase involved in cell cycle regulation and the DNA damage response, two processes which are key to myogenic differentiation.

In chapter 4, I refine the idea of checkpoint kinases controlling myogenic differentiation, and expand it to include two more checkpoint kinases, Chk1 and ATR. I develop a mechanistic explanation for how inhibition of these kinases leads to spontaneous myogenic differentiation, and present another example of DNA damage-driven differentiation.
Chapter 1
Caspases, DNA damage, and differentiation
Chapter 1: Caspases, DNA damage, and differentiation

1.1: Introduction

The differentiation programs of many cell types require the activity of apoptotic caspases (Connolly, 2014). Caspase activity plays numerous distinct roles in different systems; from cell migration and motility, to the projection and guidance of cell processes, to the rearrangement of the cytoskeleton. Given this fact, it seems to be the case that caspases are multifunctional enzymes whose overarching function is to effect large scale changes in cellular behavior; of which apoptosis is only a particularly extreme example.

It has been shown that the activity of caspase-3 is required for myogenic differentiation (Fernando, 2002). This caspase-3 activity is initiated by caspase-9 (Murray, 2008), and is restricted from proceeding to a full apoptotic phenotype through XIAP upregulation (Smith, 2009). Bcl-XL has also been implicated upstream of caspase-3 activity in myogenesis (Murray, 2008), perhaps indicating a role for mitochondrial membrane depolarization, although this has never been directly demonstrated.

The function of caspase activity in myogenic differentiation is still in dispute. Some groups argue that the role of caspases is strictly apoptotic, and that their function is to generate chemotactic signaling factors which attract myoblasts to the site of muscle injury and stimulate their proliferation and differentiation (Diagram 1A). Numerous caspase-driven signaling factors have been discovered that function like this, including prostaglandin E2 (Beaulieu, 2012; Zhao, 2006),
Sphingosine-1-phosphate (Mao & Obeid, 2008; Nincheri, 2009) and fractalkine/CX3CL1 (Truman, 2008), as well as apoptotic cells themselves (Hochreiter-Hufford, 2013).

On the other hand, other groups contend that there is a truly non-apoptotic and cell autonomous role for caspase activity. This has been established convincingly in other systems, such as spermatid individualization (Arama, 2007), as well as in keratinocyte (Weil, 1999) and erythrocyte (Zermati, 2001) terminal differentiation. The cell autonomous model postulates that in the absence of true apoptosis, cells can generate caspase activity. This activity can be restrained, either spatially or in magnitude, and used to affect large-scale cell fate choices (Diagram 1B). In reality, it seems likely that both apoptotic signaling and cell autonomous caspase activity are present concurrently. The evidence for both theories is compelling, and they are not contradictory or mutually-exclusive. Apoptotic signaling provides the mechanism by which myoblasts are awakened from quiescence and attracted to the site of fiber injury. Cell autonomous caspase activity is the mechanism by which myogenic gene activation, cytoskeletal reorganization, and other large-scale changes in cell behavior are affected.

In muscle systems, the evidence for a cell autonomous role of caspase activity is based on the accumulation of DNA strand breaks. These are generated by the apoptotic endonuclease CAD (caspase-activated DNase), and are seemingly required for myogenic differentiation (Larsen, 2010). Strand breaks have also been noted in seemingly non-apoptotic nuclei within regenerating muscle fibers, hinting at a true physiological role, and not merely an artifact of in vitro model cell lines (Dawson & Lough, 1988; Coulton, 1992; Sudo, 2009).
The function of myogenic DNA damage appears to be to activate myogenic genes. When CAD is genetically disrupted, myoblasts do not accumulate strand breaks, fail to express the myogenic genes p21 and myosin heavy chain, and a complete blockage in cell fusion is seen (Larsen, 2010). The authors propose a model in which DNA strand breaks are directed to particular sites in promoter regions of myogenic genes, thereby leading to their activation. This model is problematic since CAD has no sequence specificity other than internucleosomal double-stranded DNA (Hanus, 2008), so this theory would require a rather elaborate epigenetic mechanism to ensure that only the target genes are exposed at the time of CAD activity. To date, there is no evidence for such a mechanism.

I propose an alternative mechanism in which CAD-induced DNA damage is essentially random, and its function is to activate myogenic transcription factors by way of DNA damage response kinases. In this model, it is irrelevant which sites are targeted, only that sufficient DNA damage response complexes are recruited to activate myogenic transcription factors. This model gives rise to several predictions; first, agents which cause random DNA damage should induce myogenic fusion; second, this fusion should proceed even in the absence of upstream factors such as serum withdrawal, caspase signaling, and CAD activity; and third, DNA damage-induced differentiation should be dose-dependent, since a larger DNA damage response should lead to a larger recruitment of myogenic transcription factors.

In this chapter, I present evidence that random DNA damage is sufficient to induce myogenic differentiation.
1.2: Results

1.2.1 Myogenic differentiation can be modelled in vitro

C2C12 myoblasts are a commonly used cell line for modeling the behavior of skeletal muscle systems. Both myoblasts and differentiated myotubes derived from C2C12s are used to model the respective in vivo analogs. C2C12s were derived from the crush-injured thigh muscle of a C3H/HeJ mouse, and immortalized by serial passage (Yaffe & Saxel, 1977). In terms of genetic characteristics, C2C12s appear to be close to that of their parent strain, however C2C12s do contain one key mutation, a deletion of the entire INK4a/p19ARF locus which was probably acquired during immortalization (Pajcini, 2010), and may account for these cells ability to continually proliferate. Although the C2C12 cell line demonstrably differs from in vivo muscle systems, the key observations which I am building on (requirement for caspase activity and presence of DNA damage in regenerating myonuclei) have already been demonstrated in vivo, so I can be relatively confident that the C2C12 cell line recapitulates the relevant aspects of muscle regeneration.

Following the original published protocol, C2C12s are induced to differentiate by serum withdrawal, which in practice means replacing medium containing 10% FBS with medium containing 2% horse serum. For reasons that are not yet understood, resultant loss of serum components in the culture medium induce the cells to arrest in the G1 phase of the cell cycle. Following this, they withdraw entirely from the cell cycle into a phase known as G0 (Milasincic, 1996), and begin to express muscle-specific proteins, in particular the myogenic transcription factors myoD, myogenin, and MRF4, as well structural proteins such as myosin heavy chain and vimentin, and the key transcriptional regulator p21 (Andrés, 1996).
Once committed to the differentiation pathway, myoblasts align with one another along either their lateral or dorsal axes. Once the cells come into close proximity, they exchange vesicles containing unidentified components (Doberstein, 1997), and the cell membranes undergo fusion until both cells have merged into a single, multinucleate syncytium. This process can continue to occur until large, elongated fibers are formed. It is not known what factors delineate the maximal extent of fusion, since in every in vitro system fusion eventually reaches a peak value above which new myoblasts are no longer added to existent fibers.

The extent of myoblast fusion is typically measured using the fusion index assay. Briefly, cells were induced to fuse for the desired time; indicated here as ‘hours post serum withdrawal’. Cells were then formaldehyde-fixed, and immunostained for the myotube-specific structural element myosin heavy chain using an Alexa Fluor 488-conjugated secondary antibody. Nuclei were stained with the DNA-intercalating dye Hoechst 33342. Image fields were captured, and the fusion index was defined as the percent of nuclei found within multinucleate, myosin heavy chain (MyHC) positive cells. Mononucleate MyHC positive cells were excluded (MyHC expression is occasionally found to precede cell fusion) because such cells do not represent fusion events (Figure 1).
Figure 1: Induction of myogenic differentiation in vitro using the C2C12 myoblast cell line. Cells were seeded and allowed to grow overnight, washed three times with HBSS, then incubated for the indicated time in Differentiation Medium (DMEM + 2% horse serum + 2% penicillin/streptomycin; DM). Cells were then fixed and stained for myosin heavy chain. A: Gross morphology of C2C12 myoblasts and differentiated myotubes under brightfield. Distinct mononuclear stellate cells are visible under enlarged light microscopy (left). A cell undergoing mitosis is visible in the top-left corner. Elongated multinucleate myotubes are visible after 72 hours of differentiation (right). B: Fusion index values at each timepoint. C: representative immunofluorescence micrographs at each timepoint. Blue = Hoechst 33342, Green = Myosin heavy chain. Data represent means ± SEM of three independent experiments. Asterisks indicate significance at p < 0.05.
In my hands, fusion was apparent by 48 hours post serum withdrawal, and fusion proceeds to a peak at 72 hours (Figure 1B,C). Extra fusion is not typically seen past 72 hours, perhaps because all fusion-competent cells are used up, or because an essential component it the culture medium has been exhausted. For this reason, 72 hours was used as the experimental endpoint in fusion assays.

The maximum extent of fusion varies strongly with the passage number of C2C12 cells. C2C12 cultures progressive lose their ability to undergo myogenic fusion as they age, although they retain the ability to proliferate. Because cells were used at a range of passage numbers in this thesis, the baseline/control/vehicle fusion index may differ across experiments. I have taken care to include appropriate controls within each experiment, and to keep comparisons across experiments to a minimum.

1.2.2: Myogenic differentiation requires caspase activity

Muscle biopsy sections of regenerating fibers stain positive for active caspases, both in normal and disease conditions. Such observations were long thought to represent apoptotic processes within the differentiating fiber. Later it was shown with in vitro experiments that myoblast differentiation itself relies on the activity of caspase-3 (Fernando, 2002), as well as several upstream elements including caspase-9 and Bcl-XL (Murray, 2008).

Caspase-3 activity is typically measured using the DEVDase assay. Whole cell extracts were exposed to the fluorogenic caspase substrate Ac-DEVD-AMC. Active caspase-3 within the extract
cleaves the DEVD substrate, liberating the fluorescent molecule AMC, whose emission can be recorded with a spectrofluorimeter.

In differentiating myoblasts, caspase-3-like activity rose to a peak at 24 hours post serum withdrawal (Figure 2A). This is in agreement with the timecourse of myogenic caspase activation reported in the literature. Treatment with the pan-caspase inhibitor Q-VD-OPh abolished caspase activity. Treatment with Q-VD also strongly inhibited myogenic fusion (Figure 2B,C).
Figure 2: Myogenic differentiation is accompanied by caspase-3-like activity, which is required for cell fusion. Cells were seeded and treated as above, in the presence or absence of the pharmacological caspase inhibition Q-VD-OPh. A: Cell extracts from differentiating cells show cleavage of the fluorogenic substrate Ac-DEVD-AMC, with a peak at 24h post-serum withdrawal. B: Treatment with the pharmacological inhibitor Q-VD-OPh blocks myogenic fusion. C: Representative immunofluorescence micrographs of Q-VD-treated and –untreated cells. Data represent means ± SEM of three independent experiments. Asterisks indicate significance at p < 0.05.

There are several different pharmacological caspase inhibitors available. The most commonly used are Q-VD-OPh and Z-VAD.FMK. Of these two, QVD is more potent and has a milder toxicity.
profile (Chauvier, 2007). For this reason, QVD was used as the standard caspase inhibitor in these studies.

The exact mechanism of caspase activation during myogenesis has not yet been determined. The fact that caspase-9 and Bcl-X\textsubscript{L} are required for differentiation would seem to implicate the mitochondrial pathway, but direct evidence for mitochondrial events in myogenesis this is both scant and conflicting. Bloemberg (2014) reported that in differentiating myoblast cultures, Bax and PUMA are upregulated concomitant with caspase activity, but that other pro-apoptotic mitochondrial indices were undetectable. Shaltouki (2007) reported PUMA upregulation and cytochrome C release in differentiating myoblasts. Bax has been found to be upregulated in regenerating muscle fibers, but this appears to be cancelled out by a concurrent upregulation of the anti-depolarization protein Bcl-2 (Tews & Goebel, 1997). Murray (2008) attempted to investigate mitochondrial membrane depolarization directly using TMRE staining combined with flow cytometry, but were unable to detect TMRE loss during differentiation, nor were they able to detect cytochrome C release.

I also attempted to asses mitochondrial depolarization using an inhibitor-based approach (Annex I). The Voltage-Dependent Anion Channel (VDAC) is a key element in maintaining mitochondrial membrane potential (Madesh & Hajnóczky, 2001). During apoptosis, VDAC depolarization is required for cytochrome C release (Shore, 2009). DIDS (4,4'diisothicyanato-stilbene-2,29-disulfonylic-acid) is pharmacological inhibitor of VDAC depolarization. This compound has been shown to block cytochrome C release from mitochondria and is used to prevent apoptosis after various stimuli (Shoshan-Barmatz, 2010). DIDS induced a dose dependent decrease in myogenic
fusion, although at higher concentrations this appears to be due to cell toxicity of the compound. The mechanism of caspase-3 activation during myogenesis remains to be determined.

1.2.3: Myogenic differentiation is accompanied by DNA damage

It has long been noted in histological studies that regenerating muscle fibers contain nuclei which display substantial DNA damage (Coulton, 1992). These nuclei do not show gross apoptotic morphology, nor do they appear to be part of the inflammatory infiltrate. The fact that these cells are found within both the basal lamina and centrally within the regenerating fiber suggests that they are myoblasts, as the basal lamina is where quiescent myoblasts are localized, and activated myoblasts migrate to the site of regeneration. Farzaneh (1982) discovered that chick myoblasts accumulate DNA strand breaks during myogenic differentiation. Using density sedimentation, Farzaneh estimates approximately 200 strand breaks per nucleus. It was later shown that the myogenic DNA damage is produced as a caspase-dependent process (Larsen, 2010). The picture presented here is a mosaic of different findings from in vivo and cell line studies from different organisms. In order to tie these findings together, they must be recapitulated in a single system.

In this study, histone 2AX phosphorylation was as a surrogate marker for DNA strand breaks. H2AX phosphorylation is a commonly-used marker of double-stranded DNA breaks. In response to DNA damage, H2AX is phosphorylated by DNA damage response kinases, chiefly ATM, ATR and DNA-PK (see chapter 2). H2AX phosphorylation has been found to be associated with myogenic DNA damage (Larsen, 2010).

Myoblasts were induced to differentiate by serum withdrawal. 24 hours later, the cells were fixed and stained for phosphorylated H2AX (γH2AX). Nuclei were defined as γH2AX+ if they showed
diffuse, pan-nuclear staining. Cells with sporadic individual $\gamma$H2AX foci were not counted as positive, because basal levels of H2AX are found in a substantial proportion of healthy proliferating C2C12 cells. An accumulation of $\gamma$H2AX was detected at 24 hours post serum withdrawal, but not in cells maintained in growth medium for 24 hours (Figure 3A,B). Treatment with the pan-caspase inhibitor Q-VD-OPh inhibited the accumulation of phosphorylated H2AX.

The kinetics of H2AX phosphorylation resemble those of caspase-3 activity during differentiation, with a peak occurring at 24 hours post serum withdrawal, and sharply declining thereafter (compare Figure 3C to Figure 2A). Treatment with the pan-caspase inhibitor Q-VD-OPh similarly inhibited $\gamma$H2AX accumulation, showing that the peak of H2AX accumulation is not merely delayed, but blocked entirely.
Figure 3: Myogenic differentiation is accompanied by DNA damage. Cells were induced to differentiate, then fixed at each timepoint and stained γH2AX, a marker of double-stranded DNA breaks. A: representative immunofluorescence micrographs. GM indicates high-serum growth medium, DM indicates low-serum differentiation medium. Teal = γH2AX, Blue = Hoechst 33342. γH2AX foci are present at 24h post-serum withdrawal, but not in cells maintained in growth medium, nor in cells treated with the pharmacological caspase inhibitor Q-VD-OPh. B: Percent of nuclei scored positive for γH2AX at 24h. C: Timecourse of H2AX phosphorylation during differentiation. Data represent means ± SEM of three independent experiments. Asterisks indicate significance at p < 0.05.
The accumulation of γH2AX indicates not only the presence of DNA damage, but also of a functional DNA damage response. In chapter 2, it is shown that inhibition of the DNA damage response kinase DNA-PK blocks γH2AX accumulation, and also blocks myogenic differentiation, suggesting that a functional DNA damage response is required for myogenic differentiation. The source of this myogenic DNA damage has been reported to be the caspase-activated endonuclease CAD. It will next be tested whether CAD is required in this differentiation system.

1.2.4: The endonuclease CAD is responsible for inducing myogenic DNA damage

Larsen (2010) reported that myogenic DNA damage occurs, at least in part, through the activity of CAD (Caspase Activated DNase). This is the same endonuclease which is responsible for DNA laddering during apoptosis. In normal cycling cells, CAD is found complexed with its inhibitor, ICAD. During apoptosis (or when caspases are otherwise activated), CAD is cleaved from its inhibitor ICAD, where it is free to cleave nuclear DNA into internucleosomal fragments, forming double-stranded breaks.

In order to test whether the source of myogenic DNA damage is CAD, and whether CAD activity is required for myogenic fusion, an siRNA-mediated knockdown approach was used.

Transfection of myoblasts with siRNA constructs targeting CAD produced a marked decrease in myogenic differentiation compared to cells transfected with scramble siRNAs (Figure 4A,B). Likewise, Downregulation of CAD by siRNA also attenuated the peak of γH2AX accumulation at 24 hours post serum withdrawal (Figure 4C,D).
Figure 4: Myogenic strand breaks are induced by CAD. Cells were transfected with siRNA constructs targeting CAD or scramble siRNA constructs, and then induced to differentiate. A,B: Depletion of CAD by siRNA inhibits myogenic differentiation. There are still a number of myosin heavy chain positive cells present, but most are unfused mononucleate. C,D: siRNA depletion of CAD reduces myogenic DNA damage. Teal is γH2AX, blue is Hoechst 33342. Data represent means ± SEM of three independent experiments. Asterisks indicate significance at p < 0.05.
The fact that CAD cleavage blocked both myogenic DNA damage and myogenic fusion supports the notion that the caspase-3/CAD axis is responsible for induction of the observed strand breaks during differentiation, and suggests that this strand breakage is causal for the myogenic program.

A question that immediately presents itself is whether CAD imparts some sort of specificity to this DNA damage, perhaps through targeting particular sites within the genome; or whether random DNA damage itself can trigger differentiation, even in the absence of the upstream elements of the myogenic program, such as serum withdrawal and caspase activity.

1.2.5: Random damage is sufficient to induce myogenic differentiation

Larsen (2010) proposed that the function of CAD during myoblast differentiation is to activate muscle-specific genes. This is purported to work in a targeted, sequence specific manner; such that cleavage of particular regulatory sites upstream of myogenic genes leads to their activation. This proximity model is problematic because CAD has no particular sequence specificity. CAD indiscriminately cleaves internucleosomal double stranded DNA (Nagata, 2003). As such, there is no obvious mechanism through which CAD could impart sequence-specific cleavage.

An alternative explanation may be that the myogenic gene program is activated by way of DNA damage response kinases. All DNA damage kinases phosphorylate many cellular targets and lead to cell fate choices, for example inducing checkpoint arrest or programmed cell death. It is conceivable that differentiation is another such cell fate choice which can be made in response to DNA damage. This could be achieved through the activation of myogenic transcription factors by DNA damage response kinases. In this model, it is irrelevant where the DNA damage is localized.
within the genome, just that sufficient damage response complexes are engaged to activate myogenic signaling.

To test whether random DNA damage is sufficient to induce myogenic differentiation, DNA damage was produced directly through UV irradiation. UV in the 250 nm range is close to the maximal absorption spectrum of DNA (Elkind, 1978), which is why light in this range is typically used for germicidal applications. Cycling myoblasts maintained in growth medium were exposed to a 250 nm-peak UVC light source outputting an irradiance of 117 μW/cm².

UV irradiation of cells produced spontaneous myogenic differentiation (Figure 5A,B). Untreated cells showed minimal fusion, with a number of mononucleate myosin heavy chain positive cells. Cells irradiated for 30 and 60 seconds show much higher levels of fusion, approaching that of cells treated with serum withdrawal (DM). In some experiments, 30 and 60 second irradiation time produced levels of fusion that are equivalent to that seen with serum withdrawal. At 2.5m irradiation time, almost all nuclei showed signs of apoptosis or necrosis, and the background myosin heavy chain expression was abolished.

Treatment of cells with UV also induced a strong, rapid and dose-dependent accumulation of nuclear γH2AX (Figure 5C,D). This DNA damage response was apparent at 2.5 hours post irradiation. Such rapidity suggests that it is the direct result of UV bombardment of DNA, rather that of an indirect process. In many nuclei, the UV-induced DNA damage persisted for the duration of the experiment, as γH2AX positive nuclei were found within terminally-differentiated myotubes (Figure 5E), which is very rarely seen in serum withdrawal-induced cells.
Figure 5: Exposure to UV irradiation is sufficient to induce differentiation. A: Cells maintained in growth medium were exposed to UV at an irradiance of 117 µW/cm² for the specified time. Fusion was allowed to proceed for 72h before cells were fixed and stained for myosin heavy chain. B: Fusion index of UV-induced cells compared to cells induced by serum withdrawal. C,D: UV-irradiation produces rapid and dose-dependent accumulation of γH2AX. E: γH2AX is retained in myotubes produced by UV irradiation, but not in myotubes produced by serum withdrawal. White arrowheads point to γH2AX positive nuclei (green) within myotubes (red). Data represent means ± SEM of three independent experiments. Asterisks indicate significance at p < 0.05.
The fact that apparently random DNA damage induces myogenic differentiation seems to preclude the possibility that specific sequences proximal to myogenic genes must be cleaved to activate the differentiation program. The myogenic gene expression program is tree-shaped, with a small number of apical transcription factors such as myoD, myogenin, Myf5 and MRF4, which each turn on a large number of genes (Buckingham & Rigby, 2014). As such, there must be a comparatively small number of sites which must be ‘hit’ in order to trigger the differentiation program in the proximity model.

Given the size of the mouse genome (2.8 billion base pairs), it seems unlikely that such specific loci are hit by random bombardment with UV irradiation. Even if those sites are relatively labile, for example if anywhere within a 100 basepair region is sufficient to trigger activation of myogenic genes, there are still 28,000,000 such regions within the genome. The likelihood of hitting such target regions consistently in each of the cells which undergo fusion still seems low.

Why DNA damage is retained in UV-induced myotubes, but not in serum withdrawal-induced myotubes is still a mystery. A report from 1971 (Stockdale) indicated that myoblasts exposed to UV irradiation during differentiation engage in unscheduled DNA synthesis (i.e. DNA repair), but that myoblasts lose this ability to repair such DNA damage as they differentiate. Perhaps UV irradiation alters the ability of the cell to repair DNA damage to the same extent. It is also possible that DNA repair is unaffected, but that the normal processes which dephosphorylate H2AX have been impaired by irradiation.
1.2.6: CAD inhibition cannot block UV-induced differentiation

Above, I have presented evidence that random DNA damage is sufficient to induce myogenic differentiation. However, it could be argued that UV irradiation itself induces caspase activity, and thus canonical differentiation through the following schema:

UV irradiation > Caspase-3 activity > CAD > directed DNA damage > differentiation

In order to preclude this possibility, UV irradiation was combined with siRNA against CAD, as well as with pharmacological inhibition of caspases with Q-VD-OPh. If random DNA damage is truly sufficient to induce differentiation, then siRNA knockdown of CAD or inhibition of caspase activity should have no effect on UV induced differentiation.

Cells were either transfected 24 hours prior to irradiation with siRNA against CAD or were pretreated for 1 hour prior to irradiation with 30 µM Q-VD-OPh. After this, the cells were irradiated as above, with an exposure time of 45 seconds. Cells were then allowed to fuse for 72 hours and fixed for fusion index calculation.

Depletion of CAD by siRNA was unable to block UV-induced myogenic differentiation (Figure 6A). This suggests that UV-induced DNA strand breaks are sufficient to drive differentiation even if CAD has been inhibited. This is consistent with a model where random, not site directed, DNA damage is sufficient to drive differentiation.

Depletion of caspase activity prior to irradiation produced a decrease in myogenic fusion, but a decrease which was much lower than that seen when cells induced by serum withdrawal were treated with QVD (Figure 6B). Treatment of serum withdrawal-induced myoblasts with QVD
entirely blocked myogenic fusion. This dose of QVD is routinely used to block full apoptotic caspase activity, and has been shown to almost completely deplete caspase-3 activity in differentiating myoblasts (Bloemberg, 2014).
Figure 6: Effect of CAD and caspase inhibition on UV-induced differentiation. A: Cells were transfected with siRNA against CAD. 24 hours after transfection, the cells were washed once in growth medium, and then exposed to UV irradiation for 45 seconds. Fusion was allowed to proceed for 72h before cells were fixed and stained for myosin heavy chain. B: Cells were pre-treated for 1 hour with the pharmacological caspase inhibitor Q-VD-OPh at 30 µM. Cells were then irradiated for 45 seconds in growth medium, and allowed to fuse for 72 hours, then fixed and stained for myosin heavy chain. Data represent means ± SEM of three independent experiments.
The fact that UV irradiation was able to overcome CAD inhibition, but not caspase inhibition, suggests that caspases have more roles in myogenesis than simply inducing DNA damage. Myogenesis is a multi-faceted process which involves many different morphological changes to the cell, such as rearrangement of the cytoskeleton and the phospholipid membrane. It is conceivable that some of these other changes are also under the control of caspases.

1.3: Discussion

Here I have confirmed reports from the literature showing that caspase activity is required for myogenic differentiation. I have also shown that caspases induce DNA strand breaks through the activity of CAD, an apoptotic endonuclease, and that the activity of CAD is required for myogenic differentiation to proceed. I then showed that direct induction of DNA damage by UV irradiation is sufficient to trigger myogenic differentiation. This is the case even when CAD activity is depleted. UV irradiation can also partially rescue fusion when caspase activity is inhibited, although it is possible that UV irradiation itself induces a sustained low-level of caspase activity which is responsible for CAD-independent aspects of myogenic differentiation.

There are two possible theories to explain how CAD induced DNA damage leads to myogenic gene activation. The proximal model suggests that specific sequences are cleaved upstream of myogenic genes, leading to their activation (Larsen, 2010). Promoter-specific cleavage was detected for the cell cycle regulator p21, but not for the myogenic transcription factor myogenin. Another possible theory is that DNA damage response kinases directly activate myogenic transcription factors. In this theory, it is irrelevant which sequences are cleaved per se, only that a
sufficient DNA damage response is generated to activate myogenic transcription factors. It has been shown that the androgen receptor is directly activated by the DNA-PK complex in this fashion (Mayeur, 2005; Vlahopoulos, 2005).

The evidence presented here suggests that the second theory is more likely. If promoter-specific sequences were required to be cleaved in order to activate the myogenic program, then random DNA damage supplied by UV irradiation should not be able to trigger myogenesis. The myogenic program relies on the activation of a small number of key upstream myogenic transcription factors, principally the Myogenic Regulatory Factors (myoD, Myf5, myogenin, and MRF4), which in turn activate the wider gene transcription network (Buckingham & Rigby, 2014). In order for the promoter-specific theory to be correct, these sites would have to be hit by random bombardment with UV light. It seems unlikely that this would happen unless the genome were heavily saturated with strand breaks to the point that the cell would likely die in any case.

It has previously been reported that induction of DNA damage activates a myogenic checkpoint which blocks myogenic differentiation (Puri, 2002). The authors present this as a mechanism by which cells can avoid entering their differentiation program before genotoxic lesions are repaired, and thus protect the genomic stability of differentiated postmitotic myotubes. This appears to be at odds with the work presented here. According to Puri et al, treatment with the genotoxic agents etoposide, cisplation, and MMS all blocked myogenic differentiation. However, all three compounds have significant side effects which may account for their fusion-inhibitory effects without invoking the explanation of a damage-induced checkpoint. Some of these observations
were repeated in Annex II. A substantial decrease in myogenic fusion was detected, but this fusion-inhibitory effect was due to cell toxicity of the drugs.

With respect to UV irradiation, there appears to be a dose-dependent relationship between irradiation time and myogenic induction. Exposure of myoblasts for 2.5 to 5 minutes produces massive cell death and necrosis. Exposure for 30-60 seconds more or less recapitulates the extent of fusion normally seen with serum withdrawal. Exposure for 0-15 seconds produces no fusion whatsoever (15 seconds exposure not shown for clarity). Thus there appears to be a narrow range or ‘sweet spot’ of DNA damage which the cell can sustain, below which no myogenic fusion is triggered, and above which massive cell death occurs. This is in line with the myogenic transcription factor activation theory, where sufficient DNA damage (and consequentially a sufficiently robust DNA damage response) must be present to activate myogenic genes (Diagram 1).
Diagram 1: A model of dose-dependent DNA damage induced differentiation. In this model, low levels of DNA damage have little or no myogenic effect, because they generate a sub-threshold DNA damage response. Very high levels of DNA damage lead to programmed cell death or necrotic death. In between, there is a ‘myogenic window’ in which DNA damage leads to sufficient recruitment of DNA damage response proteins to drive the differentiation program. The activity CAD during myogenesis produces a level of DNA damage within this myogenic window.

In summary, this chapter presents the DNA damage is sufficient to induce the differentiation of myoblasts in vitro. Since this DNA damage is produced by UV bombardment, it is likely to be non-sequence specific. Because there is a dose-dependent relationship between UV exposure time and differentiation induction, I postulate that there is a ‘myogenic window’ of DNA damage in which differentiation can be induced. Below this threshold, exogenous DNA damage is unable to trigger the myogenic program, while larger amounts of DNA damage induce cell death. Currently, there is no technology to definitively enumerate the absolute number of DNA strand breaks within
nuclei, so defining this *myogenic* level of DNA damage may pose a challenge. In the general discussion, I propose a methodology to enumerate DNA strand breaks.

It has yet to be explained why DNA damage should activate the myogenic program. One possible explanation is that elements of the DNA damage response are required for transmitting a myogenic signal through their kinase activity, perhaps through activating myogenic transcription factors such as myoD. This would provide a link between caspase activity, CAD-induced DNA damage, and myogenic differentiation. In the next chapter, evidence will be presented that the DNA damage response kinase DNA-PK is required for myogenic differentiation.
Chapter 2
DNA-PK is required for myogenic differentiation
2.1: Introduction

Myoblast differentiation recapitulates many aspects of the process of apoptotic cell death. This includes features such as rearrangement of the cytoskeleton, the exposure of phosphatidylserine on the outer cell membrane, and the cleavage of DNA into internucleosomal fragments by CAD, although in all these cases the phenotype is much less pronounced during differentiation compared to apoptosis.

It has previously been shown that myogenesis involves the activity of caspases (Fernando, 2002; Ikeda, 2009). Pharmacological or genetic inhibition of caspase-3 completely blocks the process of muscle differentiation, and leads to profound growth defects in the embryo (Kuida, 1999; Woo, 1998). Likewise, inhibition of the upstream activator caspase-9 produces a similar phenotype, and the same is true if anti-apoptotic mitochondrial Bcl proteins are upregulated (Murray, 2008).

This is indicative of a wider role for caspases in cell differentiation programs (indeed, it has been postulated that apoptosis itself as just an ‘extreme’ form of cell differentiation (Fernando, 2007)). Caspases have been found to have a wide range of roles in the differentiation programs of many cell types; including the enucleation of red blood cells; the formation and dissolution of axons in neuronal cells; and in the processes of motility and metastasis (reviewed in Connolly, 2014). In general, it appears that caspases are capable of inducing large-scale morphological changes to the cell, and are involved in reshaping the cell so that it can fulfil a changing role.
Within myogenesis, caspases probably have several roles. One role that has been elucidated is the generation of DNA strand breaks. During both myogenesis and apoptosis, caspase-3 activates a nuclear DNase known as CAD. CAD cleaves internucleosomal DNA strands, forming double-stranded breaks. CAD-mediated DNA cleavage is not merely an inconsequential side effect of caspase activity, but is actually required for the myogenic program. Inhibition of CAD alone blocks myogenesis and resembles the phenotype seen under caspase inhibition (Larsen, 2010; Chapter 1 above).

The DNA damage induced during differentiation is not permanent, but transient. Analysis by TUNEL and comet electrophoresis; as well by H2AX phosphorylation, all indicate that DNA strand breaks occur concomitantly with caspase activity, rising to a peak at about 24h post-serum withdrawal, then disappearing (Larsen 2010, Connolly 2016). This seems to be indicative of a repair process, although it is also possible that the cells which accumulate DNA damage end up dying, and are thus lost to the analysis. The question presents itself: why, during differentiation, do myoblasts substantially damage their own DNA, only to immediately repair it?

It has been hypothesized that the function of CAD-induced DNA damage during myogenic differentiation is to activate gene transcription. One model that has been put forward is that CAD-induced strand breaks are formed in the promoter regions of specific myogenic genes, and that this leads to activation of these genes. The evidence for this theory is that CAD induces strand breaks into the promoter region of the myogenic gene p21, and that genetic inhibition of CAD blocks both the appearance of these strand breaks and the expression of the gene (Larsen, 2010).
An issue with this theory is that CAD has no particular sequence specificity (Hanus, 2008), and it is not obvious why CAD should target a particular cohort of genes during differentiation, and not any other. This theory seems to imply a rather elaborate epigenetic mechanism to control which sequences are made available to CAD, a mechanism for which there is currently little evidence.

Another possible explanation for the role of CAD in myogenesis is that the generation of strand breaks is used to induce the formation of DNA damage response complexes, and that kinase signaling from these DDR complexes is used to directly activate myogenic transcription factors, which in turn activate the wider myogenic program. In this model, it is irrelevant which sequences are cleaved per se, only that sufficient DDR signaling is generated to activate transcription factors. CAD-induced myogenic DNA damage is accompanied by the appearance of phosphorylated histone H2AX (γH2AX), a marker of DNA damage (Sharma, 2012). In order to determine whether myogenic gene transcription is under the control of a caspase-induced DNA damage response, we must first determine which DNA damage response in operational during myogenesis.

Canonically, H2AX is phosphorylated by members of the PI-3-related kinase family; in particular Ataxia telangiectasia mutated (ATM), ATM- and RAD3-related (ATR), and DNA-dependent protein kinase (DNA-PK). ATM plays a role in base-excision repair. ATM and ATR play role in homologous recombination, while DNA-PK is a key enzyme in non-homologous end-joining.

ATR is typically activated by replication stress. That is, it is activated in response to strand breaks induced by defects in the DNA replication. Thus, ATR activity is generally only seen during periods of active DNA synthesis, particularly in S/G2 (Lin, 2007).
In contrast, ATM is typically activated by non-replication associated strand breaks, such as those induced by ionizing radiation, free radical exposure, and other such genotoxic insults. Thus ATM is active throughout the whole cell cycle (Cuadrado, 2006).

DNA-PK is the core component of the Non-Homologous End Joining (NHEJ) pathway. NHEJ is used to repair double-stranded lesions under circumstances where a template strand is unavailable. As such, it is the dominant repair mechanism used during S/G2 (Dungl, 2015), overlapping with ATR.

Here, I set out to identify the kinase responsible for H2AX phosphorylation during myoblast differentiation, and thereby the mechanism by which caspase-mediated strand breaks are resolved. To accomplish this, I screened a panel of potent and selective inhibitors of ATM, ATR and DNA-PKcs to test which, if any, blocked myogenic histone phosphorylation. Hits from this screen were validated using siRNA, and then further characterized for their effect on differentiation and cell fusion.

This body of work was published in August 2016 in the FEBS Journal as “DNA-PK activity is associated with caspase-dependent myogenic differentiation”.
2.2: Results

2.2.1: Properties of pharmacological PIKK inhibitors

The inhibitors used in this study were NU-7441 (DNA-PK), VE-821 (ATR), and KU-55933 (ATM) (Figure 1).

![Chemical structures of the PIKK inhibitors used in this study.]

Each was chosen for its potency and selectivity relative to other PIKK inhibitors (Table 1). Although the IC\textsubscript{50} values given were derived from cell free experiments, these values should be at least a partial indication of the relative potency of each inhibitor for its given targets.
**Table 1:** Chart of targets and cell free IC$_{50}$ values for each of the PIKK inhibitors used in this study.

<table>
<thead>
<tr>
<th>Target</th>
<th>NU-7441 IC$_{50}$ (Leahy, 2004)</th>
<th>VE-821 IC$_{50}$ (Reaper, 2011)</th>
<th>KU-55933 IC$_{50}$ (Hickson, 2004)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA-PK</td>
<td>14 nM</td>
<td>13 nM</td>
<td>12.9 nM</td>
</tr>
<tr>
<td>mTOR</td>
<td>1,700 nM</td>
<td>&gt;1,000 nM</td>
<td>2,500 nM</td>
</tr>
<tr>
<td>PI3K</td>
<td>5,000 nM</td>
<td>DNA-PK 2,200 nM</td>
<td>mTOR 9,300 nM</td>
</tr>
<tr>
<td>ATM</td>
<td>&gt;100,000 nM</td>
<td>PI3K 3,900 nM</td>
<td>PI3K 16,600 nM</td>
</tr>
<tr>
<td>ATR</td>
<td>&gt;100,000 nM</td>
<td>ATM 16,000 nM</td>
<td></td>
</tr>
</tbody>
</table>

2.2.2: Effect of PIKK inhibitors on cell viability

Each of the compounds used in this study is a selective and potent inhibitor of its target kinase. In order to investigate effects on myogenic differentiation, and discount the possibility that such effect are caused simply by direct cell toxicity, the compounds were tested by the resazurin cell viability assay. The resazurin assay relies on the reduction of the blue chemical dye resazurin to the colourless compound reosurfin. This reduction is carried out by cellular reductase enzymes, and is thus a correlate of the metabolic activity of the cell. Exposure of cells to a metabolotoxic compound will inhibit the reduction of resazurin, and thus the measured loss of absorbance.

Briefly, cells were seeded into 96 well tissue culture plates 24 hours prior to compound addition. Compounds were added at a range of concentrations from 1 nM to 30 µM. Cells were incubated in the presence of the compound for three days in either differentiation medium or growth medium. At this point, resazurin dye was added to a final concentration of 93 µM. The plates were incubated for 6 hours at 37 °C in 5% CO2, and dye reduction was measured at Ex 530 nm / Em 595 nm.
Each compound elicited a dose-dependent decrease in cell viability (Figure 2). Based on these data, a screening concentration was established. The concentration chosen for screening was the highest concentration which produced no cellular toxicity; 0.1 µM in the case of the ATR inhibitor VE-821 (Figure 2A), and 1 µM in the case of both the ATM inhibitor KU-55933 and the DNA-PK inhibitor NU-7441 (Figure 2B,C).

**Figure 2: Cell toxicity profiles of PIKK compounds.** The effect of DDR kinase inhibitors on cell viability. Cells were cultured in growth medium or differentiation medium, and treated with increasing concentrations of kinase inhibitors VE-821 (A), KU-55933 (B) or NU-7441 (C). Cell viability was assessed using the reszaurin viability assay. Data represent means ± SEM of three independent experiments.
Interestingly, the DNA-PK inhibitor NU-7441 elicited a differential effect on cell viability depending on whether the myoblasts were cultured in differentiation or proliferation conditions. This appeared to indicate a specific role for DNA-PK during myogenic differentiation.

### 2.2.3: H2AX phosphorylation during myogenesis can be blocked by DNA-PK inhibition

Histone 2AX phosphorylation is a commonly-used marker of double-stranded DNA lesions. H2AX phosphorylation accompanies the caspase-induced DNA damage during myogenic differentiation (Larsen, 2010; Connolly, 2016). The role of myogenic DNA damage is unknown. I hypothesize that elements of the DNA damage response are responsible for activating myogenic genes. In order to test this, it must first be determined what kind of DNA damage response is active during myogenic differentiation.

Cells were treated with each of the three PIKK inhibitors at the concentration determined earlier, then induced to differentiate by serum withdrawal. After 24 hours (the peak of myogenic DNA damage determined in the literature and confirmed in Chapter 1 above), cells were fixed and stained for phosphorylated histone 2AX (γH2AX). Nuclei were defined as γH2AX positive if they contained a diffuse, pan-nuclear staining. Cells were not defined as positive if they contained only sporadic γH2AX foci.
Treatment with the DNA-PK inhibitor NU-7441 blocks the accumulation of γH2AX foci in differentiating nuclei, while neither treatment with the ATR inhibitor VE-821 or the ATM inhibitor KU-55933 blocked γH2AX accumulation (Figure 3C). This appears to suggest that DNA-PK plays a role in the DNA damage response during myogenic differentiation.

Myogenic DNA damage is a caspase-dependent event (Larsen; 2010). Because of this, inhibition of caspases also blocks the DNA damage response in the form of γH2AX accumulation. In order to compare the effect on γH2AX accumulation of DNA-PK inhibition relative to that of caspase inhibition, a timecourse analysis was performed. Cells were treated with either NU-7441 or Q-VD-OPh and induced to differentiate by serum withdrawal. Cells were then fixed and stained for γH2AX at the indicated timepoints. In untreated differentiating cells, γH2AX staining rises to a peak at 24 hours post serum withdrawal, before quickly decreasing by 48 hours post serum withdrawal (Figure 3B). This resembles the pattern of H2AX phosphorylation seen previously (Larsen, 2010). In cells treated with either caspase or NU-7441 inhibitors, no peak of H2AX phosphorylation was seen at 24 hours, or at any other time. Together, this appears to indicate that DNA-PK inhibition has the same inhibitory effect upon H2AX phosphorylation as does caspase inhibition.

In order to get a more fine-grained picture of the effect of DNA-PK inhibition upon γH2AX phosphorylation, the number of foci per nucleus were enumerated. In cycling cells, a low, basal level of γH2AX foci is seen (Figure 3D, GM). This probably represents the physiological DNA damage which results from normal metabolic and oxidative processes. In differentiating cells (Figure 3D, DM), a large shift is seen toward nuclei with high γH2AX accumulation (>25 foci per
nucleus). The number of such nuclei approximately doubles between cycling and differentiation cells. When cells were treated with the caspase inhibitor (Figure 3D, Q-VD-OPh) or the DNA-PK inhibitor (Figure 3D, NU-7441), this shift towards high \( \gamma \)H2AX accumulation is abolished. Treatment with the structurally-related DNA-PK inhibitor NU-7026 also abolishes \( \gamma \)H2AX accumulation (Figure 3D, NU-7026).
Figure 3: Accumulation of H2AX foci during differentiation and their inhibition by treatment with NU-7441. (A) Example micrographs of H2AX foci under various treatment conditions. (B) The effect of NU-7441 on the percentage of nuclei with > 20 γH2AX foci over time. (C) The effect of other DDR kinase inhibitors on H2AX phosphorylation. At 24 hours the number of nuclei with >25 γH2AX foci was assessed. (D) The effect of NU-7441, NU-7026, and Q-VD-OPh treatment on the accumulation of H2AX foci during differentiation. Data represent means ± SEM of three independent experiments.
The data presented here suggest that γH2AX foci accumulate during myogenic differentiation. This accumulation is caspase-dependent, and can be blocked by treatment with pharmacological DNA-PK inhibitors. Taken together, this suggests that DNA-PK is responsible for at least some aspects of the DNA damage response during myogenic differentiation.

2.2.4: DNA-PK inhibition blocks myogenic fusion

Caspase-induced DNA damage is required for myogenic differentiation. The function of this DNA damage is not yet known. One possibility is that elements of the DNA damage response are responsible for activating the myogenic program, perhaps through kinase-mediated activation of transcription factors. In the previous section, it was shown that the myogenic DNA damage response is mediated in part by the non-homologous end-joining kinase DNA-PK. If the DNA damage response is required for activating the myogenic program, then inhibiting it should also block downstream aspects of myogenic differentiation.

To test this, myoblasts were treated with each of the three PIKK inhibitor compounds and induced to differentiate by serum withdrawal. Treatment with the DNA-PK inhibitor NU-7441, but not with the ATR inhibitor VE-821 nor the ATM inhibitor KU-55933 blocked myogenic differentiation (Figure 4A,B). Additionally, treatment with both NU-7441 and the structurally-related DNA-PK inhibitor NU-7026 produced a dose-dependent inhibition of myogenic fusion (Figure 4C).
Figure 4: Inhibition of myogenic fusion by NU-7441. A: The DNA-PK inhibitor NU-7441, but not the ATR inhibitor VE-821 nor the ATM inhibitor KU-55933, blocks myogenic fusion. B: Example micrographs of fusion inhibition by NU-7441. C: Dose-response of fusion inhibition by NU-7441 and the structurally-related inhibitor NU-7026. Data represent means ± SEM of three independent experiments. Asterisks indicate significance at p < 0.05.

The data presented here suggests that the activity of the DNA damage response kinase DNA-PK is required not only for aspects of the myogenic DNA damage response, but is also required for myogenic fusion. This is in line with the theory that DNA damage response signaling is involved in the progression of the myogenic fusion program.
NU-7441 is an ATP-competitive inhibitor of DNA-PK, and a crystal structure has been solved showing NU-7441 bound in the active site of DNA-PK, suggesting that the mechanism through which NU-7441 inhibits DNA-PK is by directly blocking its kinase activity.

2.2.5: Inhibition of DNA-PK by siRNA reproduces the effect of pharmacological inhibition

The data presented above suggests that DNA-PK plays a role in myogenic fusion, but is based solely on pharmacological drugs. Although NU-7441 is a potent inhibitor of DNA-PK and has high specificity towards DNA-PK compared to other targets, it is always possible that NU-7441 exerts its myogenic-inhibitory effect through another, unspecified target.

To confirm the target validity of NU-7441, an siRNA-based approach was used. Three siRNA constructs directed against different sequences within the murine DNA-PK transcript (PRKDC) were used. Cells were transfected with the siRNA constructs 24 hours prior to fusion induction by serum withdrawal. After 72 hours of fusion, cells were fixed and stained with myosin heavy chain, and the fusion index was enumerated.

siRNA-mediated knockdown of PRKDC produced a significant reduction of myogenic fusion compared to cells transfected with a scramble siRNA (Figure 5A,B). siRNA-mediated knockdown also reduces transcript levels of PRKDC (Figure 5C).
Figure 5: siRNA inhibition of DNA-PK blocks myogenic differentiation. (A) All three siRNA constructs against PRKDC block myogenic fusion. (B) Example micrographs of each siRNA construct. (C) Confirmation of efficacy of knockdown by PCR with gel electrophoresis. Data represent means ± SEM of three independent experiments. Asterisks indicate significance at p < 0.05.

The data presented here suggests that DNA-PK is the genuine target of NU-7441, and its inhibition is responsible for the effects on the DNA damage response and myogenic fusion seen above. This
data also indicates that myogenic fusion can be blocked not only by kinase inhibition of DNA-PK but also by transcription downregulation of the *PRKDC* gene transcript. This may be of interest since there are several genetic disorder in which DNA-PK is downregulated or contains inhibitory mutations (see Discussion).

### 2.2.6: DNA-PK inhibition does not suppress caspase activity

It has been established that caspase activity is required for myogenic fusion. It has also been established that DNA-PK activity is required for myogenic fusion. This is consistent with a model in which caspase-3 induces nuclear DNA strand break by way of the endonuclease CAD, and the DNA-damage response to these breaks is mediated in part by DNA-PK, and such a response is required to activate the downstream myogenic program. However, it is also possible that the inhibition of DNA-PK itself suppresses caspase-3 activity, and that this accounts for the inhibition of myogenic fusion seen with DNA-PK inhibition. In other words, the epistasis of the relationship between caspase-3 and DNA-PK must be established.

To test this, myoblasts were incubated with either caspase or DNA-PK inhibitors, and then induced to differentiate by serum withdrawal. Cells were then harvested and the level of caspase-3 activity was assessed using the DEVDase assay. Briefly, cells were collected by trypsinization, and lysed with a detergent solution. The cell extracts were then incubated with the fluorogenic caspase-3 substrate DEVD-AMC. A 1 timecourse of fluorescence was recorded using a spectrofluorimeter (Ex 380 nm / Em 450 nm), and caspase activity was measured as the slope of the initial linear phase of the resultant plot.
Cells induced to differentiate by serum withdrawal showed a marked increase in caspase-3-like activity by 24 hours post serum withdrawal (Figure 6A, Day 1). Cells which were induced to differentiate in the presence of the caspase inhibitor Q-VD-OPh (30 µM) showed no DEVDase activity (Figure 6A, Q-VD-OPh). Cells which were induced to differentiate in the presence of either of the DNA-PK inhibitors NU-7441 or NU-7026 showed no inhibition of DEVDase activity (Figure 6A, NU7026 and NU7441).

Additionally, caspase-3 processing was assessed by western blotting (Figure 6B). In untreated cells induced to differentiate, the proform of caspase-3 is processed to its active fragments p17 and p15 by 24 hours post serum withdrawal, suggesting that autocatalytic processing of caspase-3 is blocked. In cell treated with the pan-caspase inhibitor Q-VD-OPh, processing of caspase-3 to its mature p17 and p15 forms is blocked, although a p20 subunit is present. This probably represents partial processing by caspase-9. Treatment with the DNA-PK inhibitor NU-7441 has no effect on the processing of caspase-3. The forms of caspase-3 seen are indistinguishable from those present during normal myogenic differentiation.
Figure 6: DNA-PK inhibition does not block myogenic caspase activity or processing. Cells were induced to differentiate in the presence of either a caspase or a DNA-PK inhibitor. A: Treatment with NU-7441 does not alter caspase-3-like DEVDase activity during myogenic differentiation. B: Treatment with NU-7441 does not prevent the cleavage-activation of caspase-3 during differentiation. Data represent means ± SEM of three independent experiments. Asterisks indicate significance at p < 0.05.

Taken together, the data presented here suggest that DNA-PK inhibition has no effect on caspase processing during myogenic differentiation. Because of this, the hypothesis that DNA-PK inhibition causes a retrograde inhibition of caspase-3 processing or activity can be rejected. The data here are consistent with the theory of DNA-PK-induced myogenic signaling downstream of caspase activity.
2.2.7: DNA-PK inhibition does not alter the magnitude of myogenic DNA damage

Above, I have demonstrated that the activity of DNA-PK is required for the accumulation of H2AX foci during differentiation. There are two possible explanations for this; one is that DNA-PK is genuinely required for the generation of the DNA damage response to myogenic DNA lesions; the other is that NU-7441 treatment prevents the accumulation of DNA damage itself during differentiation. In order to distinguish between these two possibilities, I have attempted to measure DNA damage accumulation directly, using the comet (single-cell gel electrophoresis) assay. This assay gives a measurement of the extent of DNA damage within the chromatin.

In brief, whole, unfixed cells are embedded into agarose plugs, and adhered to a glass microscope slide. The slides are then treated with strong alkali and denaturing solutions to unwind and depolymerize the nuclear DNA. The slides are then subject to a constant electrical field to draw nuclear DNA out of the cell and into the agarose plug along a fixed vector, producing a ‘tail’ of DNA which can be visualized with a fluorescent dye. The length of the tail is proportional to the gross number of strand breaks that were present in the nucleus at the time of harvest.

Proliferating cells show a very low level of tail formation (Figure 7, A, 0h GM). Cells which are induced to differentiate by serum withdrawal show a substantial number of strand breaks by 24 hours (24h, DM), while cells maintained in growth medium show no such accumulation of strand breaks (24h, GM), indicating that this effect is not merely an artifact of being maintained in culture.
Treatment of cells with NU-7441 along with serum withdrawal showed no difference in strand breakage when compared to untreated differentiating cells (24h, DM + NU-7441).
Figure 7: DNA-PK inhibition does not inhibit the accumulation of DNA strand breaks during myogenic differentiation. A: DNA strand breaks are accumulated by 24h post-serum withdrawal, but not in cells maintained in growth medium for 24 hours. Treatment with NU-7441 was unable to alter the magnitude of myogenic DNA damage. B: Example micrographs of each condition. (C) NU-7441 induces a small decrease in cell number by 24h post-serum withdrawal. Data represent means ± SEM of three independent experiments. Asterisks indicate significance at p < 0.05.
According to the data presented here, NU-7441 was unable to block the accumulation of DNA strand breaks during myogenic differentiation. It is important to note that this a simple measure of the magnitude of DNA damage during differentiation, and not of the rate of DNA repair. DNA-PK is a key enzyme for resectioning double-stranded breaks, and it stands to reason that interfering with its function would alter the rate of repair of myogenic DNA lesions. The purpose of this experiment was to test whether the decrease in H2AX foci accumulation observed under DNA-PK inhibition could be attributed to a decrease in the accumulation of DNA damage due to an unexpected effect of the inhibitor. The data appears to sustain the argument that this is not the case.

2.3: Discussion

In this section, I have presented evidence that the activity of DNA-PK is required for myogenic differentiation. DNA-PK appears to be, in part, responsible for engaging the DNA damage response to caspase-induced myogenic DNA damage. Additionally, inhibition of DNA-PK also blocks cell fusion to a similar degree seen under caspase inhibition. I have ruled out the possibility that DNA-PK alters myogenic caspase activity, nor does it alter the magnitude of DNA damage which accrues by 24 hours post serum withdrawal, which seems to indicate that DNA-PK inhibition has no effect on CAD endonuclease activity. Together, this data suggests a sequence of events as depicted in Diagram 1.
Diagram 1: Proposed role of DNA-PK in myogenic differentiation. A schema in which the DNA-PK complex acts as a phosphorylation source for factors which are involved in the myogenic program.
In this pathway, the trigger for myogenic differentiation activates caspase-3. Caspase-3 then activates the endonuclease CAD, which cleaves internucleosomal DNA, creating double stranded DNA breaks. These strand breaks are quickly bound by Ku70/80 (it has been shown through micro-irradiation studies that Ku70/80 binding to DNA is very rapid; it is detected within two seconds of irradiation of fibroblasts (Britton, 2013)). Ku70/80 recruits the kinase DNA-PKcs (and probably other members of the non-homologous end-joining complex). The DNA-PK complex phosphorylated histone 2AX, indicating a functional canonical response to double-stranded breaks. DNA-PK may also phosphorylate other targets, as it does during non-homologous end-joining, such as mdm2, a negative regulator in p53 that has been implicated in myogenesis (Smith, 1999; Fu, 2015).

Myogenic differentiation seems to proceed as a result of DNA-PK activity, as evidenced by the very strong inhibition of terminal cell fusion seen under DNA-PK inhibition. It is tempting to speculate that the role of DNA-PK in myogenesis is to phosphorylate and activate myogenic transcription factors. This could be thought of as a mechanism by which caspases can affect gene expression changes, thereby altering cell fate. I do not as yet have enough evidence to sustain this argument, although interestingly, it has been shown that the trimeric Ku/DNA-PKcs complex can activate the androgen receptor, a major myogenic transcription regulator (Mayeur, 2005; Vlahopoulos, 2005). This activation can apparently proceed even in the absence of ligand. This could represent one mechanism through which DNA-PK engages the myogenic program.
Interestingly, it has been shown that committed myoblasts have higher expression of DNA-PK relative to quiescent satellite cells, and this is accompanied by a higher NHEJ repair activity (Vahidi Ferdousi, 2014). Fully differentiated myotubes have both lower DNA-PK expression and lower repair activity compared to myoblasts (Vahidi Ferdousi, 2014). The fact that there is an increase in DNA-PK activity that is restricted to fusion-competent myoblasts suggests a particular role for DNA-PK during this stage of myoblast commitment, and is in line with the data presented here which suggests a role for DNA-PK in terminal differentiation.

Relevance of these findings in vivo

It was shown above that pharmacological inhibition or siRNA-mediated knockdown of DNA-PK prevents myogenic differentiation. These findings are restricted to an in vitro tissue culture model of myogenic differentiation. If this represents a true role for DNA-PK in muscle development in vivo, then the implication is that reduced DNA-PK activity should lead to impaired muscle development or regeneration in vivo.

A major role for DNA-PK is found in V(D)J recombination, in which gene segments are combined to produce diverse antibody coding sequences in B lymphocytes. There exist numerous severe medical disorders which have at their root loss of V(D)J recombination activity. Chief among these is Severe Combined Immunodeficiency (SCID). SCID is a congenital disorder in which the individual has severely reduced immune function, which require constant medical supervision and monitoring, frequent (if not permanent) hospitalization, and leads to a very short life expectancy. In this chapter, it was shown that DNA-PK seems to be required for myogenic differentiation, at
least *in vitro*. If this is representative of a true involvement of DNA-PK in muscle development, then SCID animals should have inhibited muscle development or regeneration.

The most commonly used animal model of SCID is the SCID mouse (B6.CB17-Prkdc<sup>scid</sup>/SzJ). It has been shown that SCID mice have apparently normal muscle regeneration capacity (Cousin, 2013). This seems to suggest that DNA-PK is irrelevant for myogenic differentiation, or that its role is redundantly carried out by other mechanisms. However, on closer examination, the SCID mouse is an imperfect model of DNA-PK deficiency.

SCID mice are not true knockouts of DNA-PK. This strain arose as a spontaneous mutation in a B6.CB17 litter (Bosma, 1983). They harbor a nonsense mutation in the non-catalytic C-terminal region of DNA-PK (Blunt, 1996). This mutation leads to a large decrease in the protein levels of DNA-PK, but it is still detectable, and still harbors a functional catalytic domain (Kirchgessner, 1995; Peterson, 1995; Araki, 1997; Danska, 1996). It is conceivable that SCID mice retain enough active DNA-PK to perform muscle development and regeneration, but not enough to perform immunogenic recombination. There may also differences in tissue expression of DNA-PK between skeletal muscle and immune cells in SCID mice. That being said, SCID mice are smaller than their isogenic littermates (Reiling, 2014), and they have a scrawny, emaciated appearance (Espejel, 2004), although this could be due to any number of factors associated with this severe disease.

It remains to be seen whether true DNA-PK deficiency would impair muscle development in mice. A useful experiment in this regard might be to administer the DNA-PK inhibitor NU-7441 to mice in order to artificially deplete DNA-PK activity. NU-7441 has been administered to mice at various
at various concentrations and durations (Zhao, 2006; Ghonim, 2014), and shows good tissue
distribution and pharmacokinetic properties in mice (Zhao, 2006), however no data is available
regarding the effect of this compound on the development, regeneration, or composition of skeletal
muscle.

SCID and muscle development in humans

There is little evidence to suggest that human SCID patients have reduced muscle mass or
regeneration capacity, although muscle hypotonia is a noted symptom of the disease (Vickers,
2009). A problem with trying to draw conclusions about the role of DNA-PK in humans from
SCID patients is that SCID is caused fundamentally by the absence of antibody generation
capacity, and this can be caused by a wide range of factors, not limited to DNA-PK deficiency.
SCID is divided into several disease subgroups based on their etiology and molecular pathology
(Table 2). The failure of antibody generation can be caused by a number of molecular mechanisms
including mutations in the gene encoding interleukin receptor gamma chain (IL-2Rγ), disorders of
dNTP synthesis or utilization such as adenosine deaminase deficiency, or through disorders of the
V(D)J recombination system, which includes DNA-PK. Because of this, an individual may have
SCID disorder without DNA-PK deficiency. As such, it is very difficult to draw conclusions about
the role of DNA-PK from analyzing data from SCID patients.
Table 2: Molecular etiology of SCID disease subgroups and approximate numbers of patients characterized with each type to date. Adapted and expanded from Kalman (2004) & Li (2002).

<table>
<thead>
<tr>
<th>Etiology</th>
<th>Disease subgroup</th>
<th>Gene affected</th>
<th>Locus</th>
<th>Patients Identified</th>
</tr>
</thead>
<tbody>
<tr>
<td>Disorders of the common gamma chain ($\gamma_c$)</td>
<td>X-Linked SCID</td>
<td>IL-2R$\gamma$</td>
<td>Xq13.1</td>
<td>169</td>
</tr>
<tr>
<td></td>
<td>JAK-3 mutation</td>
<td>JAK-3</td>
<td>19p13.1</td>
<td>27</td>
</tr>
<tr>
<td>Disorders of dNTP synthesis/utility</td>
<td>Adenosine Deaminase Deficiency</td>
<td>Adenosine deaminase</td>
<td>20q13.11</td>
<td>54</td>
</tr>
<tr>
<td></td>
<td>Purine nucleoside phosphorylase deficiency</td>
<td>PNP</td>
<td>14q22</td>
<td></td>
</tr>
<tr>
<td>Disorders of V(D)J recombination</td>
<td>Omenn Syndrome</td>
<td>RAG-1, RAG-2</td>
<td>11p13</td>
<td>62</td>
</tr>
<tr>
<td></td>
<td>Artemis deficiency</td>
<td>DCLRE1C</td>
<td>10p13</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>DNA-PKcs deficiency</td>
<td>PRKDC</td>
<td>8q11.21</td>
<td>1 (van der Burg, 2009)</td>
</tr>
</tbody>
</table>

Of all SCID patients characterized so far, only one individual has been identified with a true mutation in DNA-PKcs itself (Van der Burg, 2009). This patient bears two mutations in the PRKDC gene: a deletion of a glycine at residue 2113, and a substitution of a leucine with an arginine at residue 3062. Neither mutation lies within the kinase domain. Furthermore, experiments on tissue from this individual reveal that their DNA-PK is still kinase active, and is capable of binding Ku and of recruiting Artemis, another member of the NHEJ complex. Because of the seeming functionality of DNA-PK in this patient, it seems possible that this patient’s SCID syndrome does not actually arise from their DNA-PK mutations at all, but from some other flaw in antibody generation.

The extremely low incidence of DNA-PK deficiency among SCID patients may itself suggest that loss of DNA-PK may be embryonic or perinatal lethal in humans. That other dysfunctions of the antibody generation system can be ‘tolerated’ in the form of SCID syndrome suggests that DNA-PK itself may have functions separate from antibody generation that are required for human life.
Interestingly, DNA-PK inhibition has been picked up as a clinical target for chemotherapy, with the rationale that NHEJ inhibition should sensitize cells to radiation therapy and DNA damage-inducing drugs. Three DNA-PK inhibitors are currently being investigated in phase I clinical trials: MSC2490484A, CC-122 and CC-115 (Merck, 2014; Celgene, 2014; Celgene, 2011). These studies may shed light on whether DNA-PK has a role in myogenic development in humans.

In summary, the data presented in this chapter suggests that DNA-PK is required for myoblast terminal differentiation \textit{in vitro}, and that this activity fits with a model of caspase-induced gene regulation as part of a myogenic cell fate decision. In the next chapter, a screen of cellular kinase inhibitors is performed in an attempt to identify novel factors involved in myogenesis.
Chapter 3

Inhibition of the checkpoint kinase Wee1 strongly enhances myogenic fusion
Chapter 3: Inhibition of the checkpoint kinase Wee1 strongly enhances myogenic fusion

3.1: Introduction

Although we know much about the process of myoblast activation and the wider process of skeletal muscle regeneration, our knowledge is still far from complete. This is evidenced by the lackluster performance of myoblast implantation therapies aimed at ameliorating the effects of congenital myopathies and dystrophies (Miller, 1997; Neumeyer, 1998; Menasché, 2008; Briggs, 2013). We still do not know enough about this process to take advantage of it for medical benefit.

One of the best methods of discovering new properties of biological systems has been to use inhibitory drug screening (or its genetic correlate; screening with siRNA and shRNA libraries). Such an approach is not hypothesis-driven, and thus is not shackled to previous research or to the current understanding of biological systems. Such a ‘shotgun’ approach allows us to discover new properties that might not be suggested by previous research.

Here, I have applied a screening approach in an attempt to uncover novel kinases involved in myogenic differentiation. The compound library I have used for this screen is the Tocris Kinase Inhibitor Toolbox. This library of 88 compounds is designed to target a wide range of cellular targets. Most, but not all, of these targets are kinase enzymes. The Tocris library has an advantage over natural product libraries in that it is composed of unimolecular samples which are all well-characterized and of a defined concentration. They have been studied in previous literature, and
their pharmacological properties are well known. As such, no fractionation or purification studies need be conducted in order to identify hit compounds.

The relative simplicity of the fusion index assay used in previous chapters allows for its application to large scale screening. Hit compounds are defined as those which produce a statistically-significant change in fusion index relative to myoblasts treated with the vehicle control. Once hit compounds have been identified from a general screen, each hit must be recapitulated in isolation, to see if the effect is still present, and not simply an artifact of the screening methodology or the statistical method used. After this, hit compounds of interest are validated by an orthologous approach, such as siRNA inhibition. Only then has a true physiological ‘hit’ been uncovered.

In this chapter, I screened the Tocris kinase inhibitor library against myogenic fusion. This screen yielded six hit compounds. One hit compound; PD-407824, a pyrrolo[3,4]carbazole, was further investigated because its purported target, Wee1, is a checkpoint kinase involved in cell cycle regulation and the DNA damage response. These are two processes which are key to myogenic differentiation.

I show that Wee1 inhibition induces spontaneous and precocious myogenic fusion, even in cells maintained in growth medium. It has long been postulated that the primary factor controlling the initiation of myogenic differentiation in vitro is the withdrawal of growth factors from the culture medium through serum withdrawal. The data here suggest that myogenic differentiation can be triggered even in serum-rich medium, and that the primary trigger of differentiation lies internally within the myoblast cell itself.
Myotubes produced by Wee1 inhibition are almost double in size and nuclear number compared to those produced by conventional serum withdrawal-induced myotubes. This fusion is preceded by massive nuclear DNA damage, as measured by histone 2AX phosphorylation. This strand breakage occurs at a much earlier timepoint that that seen in ordinary, serum withdrawal-induced myoblasts, being measurable at 2.5 hours post drug addition, compared to the peak of strand breakage at 24 hours post serum withdrawal. The onset of cell fusion also occurs at an earlier time in PD4-treated myoblasts, with nascent myotubes visible after 24 hours rather than 48 hours. Taken together, the data presented here suggests that myoblasts are sensitive to perturbations in the cell cycle, and that such disruptions lead to substantial DNA strand breakage through an unknown mechanism (that will be investigated in chapter 4), and that this disruption leads to the onset of precocious myogenic fusion.
3.2: Results

3.2.1: Kinase inhibitor library screen

The kinase inhibitor library screen was performed using the fusion index assay shown earlier. Myoblasts were seeded in 96 well plates 24 hours prior to the start of the experiment. At this point, cells were exchanged into low-serum fusion medium, and drugs were added. Following this, the cells were allowed to fuse for 72 hours, before being fixed and stained for myosin heavy chain. The fusion index was enumerated as above. The drug screen was performed at a fixed concentration of 1 µM.

There are two factors which go into calculating the fusion index: the total number of nuclei counted per microphotographic field, and the number of those nuclei which are localized within multinucleated myotubes. When the cell number and the fusion index are plotted on a scattergraph, this allows the separation hit compounds which cause true fusion defects from those which simply causes cell toxicity or cell death (Figure 1).

Most compounds of the screen had little effect on myogenic fusion. This corresponds to the cluster of datapoints in Figure 1. Several compounds are statistically-significant outliers, and these correspond to hits, although some hits can be attributed to general cell toxicity. If a compound shows a reduction in fusion index in the absence of a reduction in cell number, it is taken as a true hit, in that it has an effect upon cell fusion independent of cell toxicity. If a compound shows a reduction in fusion index, but is accompanied by a corresponding reduction in cell number, the effects of that compound can likely be attributed to cytotoxicity.
Figure 1: Tocris kinase inhibitor library screen. Myoblasts were treated with each of 88 trial compounds, as well as with DMSO vehicle control. Cells were then allowed to fuse for 72 hours in the presence of drug or vehicle. At the endpoint, cells were paraformaldehyde-fixed and immunostained for myosin heavy chain. The nuclear DNA was stained with Hoechst 33342. The fusion index was scored as described above. Data is presented as the fusion index (%) versus the cell number counted per field. Data represent means ± SEM of three independent replicates.

The fusion screen yielded seven compounds which showed a statistically-significant from the control-treated cells, although one of these compound was clearly attributable to cell toxicity.
(Figure 1, data point at 500 cells/field). This suggested six compounds which were taken forward for analysis. The six compounds and their reported targets were the following:

- API-2 (Akt/PKB)
- ER-27319 (Syk kinase)
- GW-583340 (EGFR)
- Ryuvidine (SETD8-PKMT)
- PD-407824 (Wee1)

3.2.2: Hit compound characterization

In a large drug screen, pseudo hit compounds can emerge as an artifact of the statistical methods used, or from other technical issues associated with manipulating a large number of wells. Therefore, it is important to re-screen any hit compounds in isolation. On the basis of the screen above, six compounds were selected for re-screening in isolation. The fusion index assay was performed as described above.

Each of the six compounds recapitulated their fusion effect seen in the primary screen (Figure 2). For one compound, SB-218078 (an inhibitor of cdc2), the effect upon fusion was clearly attributable to cytotoxicity (Figure 2B,C). This compound corresponds to the data point at 740 cells/field in Figure 1. Interestingly one compound, PD-407824 (an inhibitor of Wee1) actually increased cell fusion. The fusion index was almost double that seen in vehicle-treated cells (Figure 2A,B).
Figure 2: Hit compounds from kinase inhibitor library screen. Each hit compound was re-screened independently for effects upon fusion index. A: Fusion index was calculated as described in earlier chapters. Each hit compound showed a significant change in fusion compared to control. B: Example immunofluorescence micrographs for each drug treatment. C: The effect on fusion index can be attributed to cell toxicity for the compound SB-218078, but not for the other hit compounds. Data represent means ± SEM of three independent experiments. Asterisks indicate significance at p < 0.05.
The primary targets and reported IC$_{50}$ values for each hit compound are outlined in Table 1 below.

**Table 1: Reported targets and IC$_{50}$ values for each hit compound.**

<table>
<thead>
<tr>
<th>COMPOUND</th>
<th>TARGET(S)</th>
<th>IC$_{50}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>RYUVIDINE</td>
<td>SETD8-PKMT</td>
<td>500 nM (Blum, 2014)</td>
</tr>
<tr>
<td>API-2</td>
<td>Akt/PKB</td>
<td>130 nM (Yang, 2004)</td>
</tr>
<tr>
<td>ER-27319</td>
<td>Syk</td>
<td>100 nM (Luskova, 2004)</td>
</tr>
<tr>
<td>GW-583340</td>
<td>EGFR</td>
<td>10 nM (Gaul, 2003)</td>
</tr>
<tr>
<td>PD-407824</td>
<td>Wee1</td>
<td>97 nM (Palmer, 2006)</td>
</tr>
<tr>
<td>SB-218078</td>
<td>Cdc2</td>
<td>250 nM (Chen, 2006)</td>
</tr>
</tbody>
</table>

Five of the six hits are attributable to non-cytotoxic effects on myogenic differentiation. SB-218078 (an inhibitor of cdc2/cdk1) shows substantial cytotoxicity at 1µM and so was excluded from further consideration.

Each of these hit compounds will be investigated in turn below for interest and novelty.

Ryuvidine targets SETD8. SETD8 is not a kinase, but a histone-lysine methyltransferase which methylates histone H4 on N-terminal tails (Couture, 2005). Histone lysine methylation is important for epigenetic control of gene transcription (Sims III, 2003). Interestingly, histone H4 lysine methylation has been implicated in the DNA damage response. Disruption of H4 lysine 20 methylation strongly impairs cell survival upon treatment with DNA damaging agents (Sanders, 2004). This may suggest that a functional DNA damage response is required for differentiation.
API-2 targets Akt/PBK. This is a ubiquitous kinase which is at the center of many cell transduction pathways. Indeed, Akt has already been implicated in myogenesis; it has been shown to interact with p21 and is involved in the process of cell cycle withdrawal (Fujio, 1999). As such, this hit is neither novel nor interesting.

Syk has not previously been implicated in myogenesis. Syk (spleen tyrosine kinase) is primarily involved in intracellular signal transduction from the B-cell and T-cell receptors (Seda, 2015), and syk knockout mice suffer embryonic lethality due to abnormal development of the hematopoietic system (Böhmer, 2010). Interestingly, one intracellular outcome of Syk is actin depolymerization and cytoskeletal reorganization, through activation of VAV and ADAP (Berton, 2005). Conceivably, Syk signaling could be involved in the cytoskeletal events that enable cell migration and alignment prior to fusion. Interestingly, Syk has been shown to interact with galectin, an important component of the myoblast migration system (Fulcher, 2009; Grossi, 2011). Taken together, this finding may suggest a previously unknown role for Syk in myoblast cell differentiation.

GW-583340 represents an interesting hit because this compound is structurally very similar to the chemotherapeutic compound Lapatinib. Lapatinib is an EGFR tyrosine kinase inhibitor which is very commonly used to target solid tumors. That GW-583340 has such a profound effect on myogenesis suggests that the related drug Lapatinib may have previously uncovered side-effects in patients, perhaps related to chemotherapy-associated weight loss. This hit compound was followed up in Annex IV.
PD-407824 is the most interesting hit compound from this screen. Rather than inhibit myogenesis, treatment with PD-407824 produces a large increase in myogenic fusion. PD-407824 is an inhibitor of Wee1 kinase. Wee1 blocks the activity of cdk1/Cyclin A complexes, and thereby maintains cells at the G2/M transition. Wee1 represents an interesting target because it is involved both in the DNA damage response (Domínguez-Kelly, 2011; Sørensen, 2012) and in cell cycle progression (Tominaga, 2006; Kellogg, 2003). These two processes are instrumental to myogenic fusion. Because of this, I chose to further investigate PD-407824.

3.2.3: PD-407824 is an inducer of myogenesis

PD-407824 is a pyrrolo[3,4-c]carbazole compound which functions as an ATP-competitive inhibitor of Wee1 kinase (Squire, 2004). Wee1 is a checkpoint kinase which controls the G2/M transition. Active Wee1 prevents transition into mitosis by phosphorylation of CDK1/cyclin B (O’Farrell, 2001). The purpose of this system is to prevent from proceeding into mitosis until the genome can be scanned for DNA damage and any damage detected can be repaired.

To test the effect of PD-407824 on myogenesis, cells were treated with a range of concentrations of PD-407824 and induced to differentiate by serum withdrawal. PD-407824 enhances myogenic fusion in a dose-dependent manner (Figure 3A). As seen earlier, the myotubes are larger, with more nuclei within each fiber (Figure 3B,C).

Between 1 and 3 µM, there is a marked decrease in cell number and fusion index (Figure 3A, 3 µM). Cells also take on the features of necrosis when treated with 3 µM PD-407824 (Figure 3D),
with many swollen and fractured nuclei. These features are absent when cells are treated with 1 µM.

Inhibition of Wee1 by siRNA recapitulates the effect on fusion seen under PD-407824 treatment (Figure 3E, panel B). This suggests that effects of PD-407824 treatment are due to inhibition of Wee1 kinase, rather than the result of some off-target effect.
Figure 3: Effect of PD-407824 on myogenic differentiation. A,B: PD-407824 exerts a dose-dependent effect on myogenic fusion, although cell toxicity rapidly sets in at concentrations higher than 1 µM. C: PD-407824 increases the average width of myotubes as measured along the lateral axis. D: Onset of cytotoxicity between 1 µM and 3 µM. Hoechst nuclear staining reveals many oversized, necrotic, and karyohectic nuclei are present. A minority of nuclei are also pyknotic. E: Effect of siRNA inhibition of Wee1 on cell fusion. siRNA “B” recapitulates the increase in fusion index and the phenotype of enlarged myotubes. Data represent means ± SEM of three independent experiments. Asterisks indicate significance at p < 0.05.
Taken together, the data here suggests that PD-407824 has a stimulatory effect upon fusion. Treatment with PD-407824 produces larger, thicker myotubes. This fusion effect is dose-dependent, but cell toxicity sets in precipitously at concentrations higher than 1 µM. This suggests a rather narrow threshold within which the fusion effect occurs, but above which cell death ensues. Whatever effect PD-407824 has upon myoblasts, that effect suddenly becomes very cytotoxic within a narrow concentration range.

3.2.4: PD-407824 induces spontaneous myogenic fusion

Treatment with the Wee1 inhibitor PD-407824 increases the fusion index of differentiating cells. There are two possible explanations for this: one is that Wee1 inhibition enhances differentiation in cells which are already committed to fuse; the other is that Wee1 inhibition itself triggers the differentiation program. If the latter is true, then Wee1 inhibition should be able to trigger myogenic fusion even in the absence of serum withdrawal.

Treatment with PD-407824 induces myogenic fusion even when myoblasts are maintained in growth medium (Figure 4A). This seems to indicate that that Wee1 inhibition can initiate myogenic differentiation, rather than simply enhance a differentiation program which is already ongoing. As seen previously, treatment with PD-407824 also enhances fusion in serum withdrawal-induced myoblasts (Figure 4B). The kinetics of fusion are increased such that the extent of fusion normally seen at 48 hours post serum withdrawal is seen at 24 hours, and the extent of fusion normally seen at 72 hours is seen at 48 hours. In other words, the rate of fusion appears to be increased by approximately one day. The maximal extent of fusion at 72 hours, the end point of the experiment, was approximately twice that seen in untreated cells.
Figure 4: PD-407824 induces spontaneous myogenic fusion, and fusion proceeds at a faster rate in serum withdrawal-induced cells. A: Myoblasts were treated with 1µM PD-407824 in growth medium and incubated for 72 hours. Cells were then fixed and stained for myosin heavy chain, then the fusion index was enumerated as described above. Myogenic fusion occurs in cells treated with PD-407824, but not in cells maintained in growth medium. B: Myogenic fusion increases the rate of fusion in cells induced to differentiate by serum withdrawal. Cells were induced to differentiate by serum withdrawal in the presence or absence of 1 µM PD-407824. Data represent means ± SEM of three independent experiments. Asterisks indicate significance at p < 0.05.

The data presented here suggests that disruption of the checkpoint kinase Wee1 induces myogenic differentiation even in the absence of serum withdrawal. Fusion also proceeds at a faster rate than is normally seen in serum withdrawal-induced cells, such that the extent of fusion normally seen at a given timepoint is instead seen 24 hours earlier. Taken together, this suggests a scenario in which Wee1 inhibition provides a stimulus that is normally present at 24 hours post-serum withdrawal, such that the earlier part of the myogenic program is circumvented.
3.2.5: *Rapid induction of DNA damage upon PD-407824 inhibition*

From the data in chapter 1 and 2, it is clear that DNA damage is associated with myogenic differentiation, and that this DNA damage occurs as a transient peak at 24 hours post serum withdrawal. Wee1 inhibition provides a stimulatory effect on myogenesis which essentially ‘speeds up’ myogenesis by one day. Because of this, I hypothesized that PD-407824 treatment induces DNA damage, and thereby directly induces myogenic fusion.

Myoblasts were treated with 1 µM PD-407824 in growth medium, as before. At the specified timepoints, cells were fixed and stained for γH2AX to reveal DNA damage. Treatment with PD-407824 produced rapid and massive nuclear DNA damage. (Figure 5A) This damage was apparent by 2.5 hours post-treatment, and gradually increases over the course of the experiment. Cells maintained in growth medium showed minimal γH2AX staining, while myoblasts induced to differentiate by serum withdrawal only show DNA damage by 24 hours. The extent of DNA damage seen in PD-407824 treated cells at 2.5 hours resembles that seen in serum withdrawal-induced cells by 24 hours.
Figure 5: Wee1 inhibition causes rapid induction of DNA damage. Cells in growth medium were treated with 1 µM of PD-407824, then fixed at the indicated time point and stained for γH2AX. Untreated cells in growth medium and cells induced to differentiate by serum withdrawal were used as controls. A,B: The DNA damage induced by Wee1 inhibition is apparent by 2.5 hours post-treatment, and gradually increases to 24 hours post treatment. In cells induced to differentiate by serum withdrawal, DNA damage is not significantly different to cells maintained in growth medium until 24 hours post serum withdrawal. Blue: Hoechst 33342, Teal: γH2AX. Data represent means ± SEM of three independent experiments. Asterisks indicate significance at p < 0.05.
In this section, it was shown that treatment with the Wee1 inhibitor PD-407824 causes a rapid induction of histone 2AX phosphorylation, indicative of DNA strand breaks. These strand breaks are detectable at timepoints soon after drug addiction, and the quantity of H2AX phosphorylation resembles that seen at 24 hours post serum withdrawal.

Together, these observations suggest a model where Wee1 inhibition produces DNA damage, and the response to that damage directly induces myogenic fusion. In essence, the caspase > CAD mechanism is circumvented and myogenic DNA damage is provided directly. This is similar to the myogenic effect of UV-induced DNA damage shown in chapter 1.

That PD4 induces myogenesis up to 1 µM, but at higher concentrations produces cell death with a rather narrow threshold between these two responses is concordance with the ‘titration model’ of DNA damage-induced myogenesis outline in the discussion of chapter 1.
3.2.6: Caspase inhibition is unable to block PD4-induced strand breaks

In the preceding sections, it was shown that treatment of proliferating myoblasts with the Wee1 inhibition PD-407824 induced a rapid accumulation of DNA strand breaks, as measured by γH2AX foci. During normal myogenic differentiation, caspase-3 activity leads to DNA strand breakage through the activity of CAD. It is possible that PD4 is a caspase inducer, and treatment with PD4 leads to strand breakage through the following schema:

PD-407824 > Caspase-3 > CAD > strand breaks > differentiation

If this is true, then pharmacological inhibition of caspase activity should be able to block the formation of strand breaks seen under PD4 treatment. If strand breaks are important to the myogenic program, as the earlier data suggests, then caspase inhibition should also block the spontaneous myogenic fusion which is seen under PD4 treatment.

Cells were treated alone with 1 µM PD4, or co-treated with 1 µM PD4 and 30 µM of the caspase inhibitor Q-VD-OPh. Treatment with PD-407824 induced substantial DNA damage by 6 hours (Figure 6D), as shown before. Co-treatment with QVD was unable to block this effect, suggesting that PD4 induces DNA damage through a caspase-independent mechanism. Treatment with PD4 enhances myogenic fusion, as seen before (Figure 6B,C), while co-treatment with PD4 and QVD is able to rescue QVD-inhibited fusion (Figure 6B,C). The fact that QVD-inhibited fusion can be rescued by PD4 treatment suggests that the myogenic effect of Wee1 inhibition can overcome caspase inhibition.
**Figure 6: PD4 can rescue Q-VD-OPh inhibited differentiation.** A: Caspase-inhibition was unable to block PD-407824-induced H2AX phosphorylation (cells fixed at 6 hours post treatment). B: Cells were co-treated with 1 µM PD-407824 and 30 µM Q-VD-OPh. QVD-inhibited fusion can be partially rescued by PD4 treatment. C: Representative immunofluorescence micrographs of treated or co-treated cells at 72 hours post serum withdrawal. Data represent means ± SEM of three independent experiments. Asterisks indicate significance at p < 0.05.
The data in this section suggests that the DNA damage induced by Wee1 inhibition is not dependent upon caspase activation. This suggests that PD4-induced strand breaks through some mechanism other than the apoptotic caspase-3 > CAD pathway. The fact that PD4 is able to rescue QVD-inhibited fusion suggests that Wee1 inhibition induces myogenesis in a caspase-independent fashion, perhaps by directly activating some downstream aspect of the myogenic program. In chapter 1, it was shown that UV-induced DNA strand breakage appears to be sufficient to induce the myogenic program. Earlier, it was shown that PD4 also causes DNA strand breaks (through a mechanism that will be investigated in the next chapter). One explanation for the myogenic effect of PD4 is that, like UV, these strand breaks drive the myogenic program; effectively bypassing the earlier phases of the myogenic program, such as serum withdrawal. This would explain the fact that myogenesis proceeds at a faster rate.

3.3: Discussion

In this chapter, the Tocris kinase inhibitor library was screened in an attempt to find novel kinases involved in myogenic differentiation. Out of 88 trial compounds, seven showed statically-significant effects upon myogenic fusion. Two of these hit compounds were attributable to cell toxicity at 1 µM. The five remaining hit compounds were the following:

API-2 (Akt/PKB)
ER-27319 (Syk kinase)
GW-583340 (EGFR)
Ryuvidine (SETD8-PKMT)
PD-407824 (Wee1)
PD-407824 was the most interesting hit from this screen, both because of the positive effect it has upon myogenesis and because of the properties of its molecular target, Wee1, which is important both in the DNA damage response and in cell cycle progression. Treatment of myoblasts with PD4 produces markedly oversized myotubes and approximately doubles the fusion index. Treatment with PD4 also induces rapid, massive DNA damage, as well as inducing spontaneous myogenic fusion in the absence of serum withdrawal. Treatment with caspase inhibitors was unable to block PD4-induced DNA damage. QVD-inhibited fusion can be rescued by PD4 treatment, suggesting that Wee1 inhibition can compensate for the myogenic signal normally provided by caspase-3.

Taken together, this is consistent with a model where caspases are required to provide myogenic DNA damage, but are also required for other functions. PD4 treatment is able to supply DNA damage when caspases are inhibited. This appears to be more evidence for the theory that random, non-sequence specific DNA damage is capable of inducing myogenic differentiation- it seems unlikely that Wee1 dysregulation and CAD inhibition provide the same cohort of DNA lesions necessary to drive fusion in a sequence specific manner.

Interestingly, cell cycle profiling has shown that Wee1 levels decrease during myogenesis (Sarkar, 2010). Wee1 is also transcriptionally-downregulated during the first day of differentiation (Shen, 2003). Wee1 is a substrate of caspase-3 during apoptosis (Zhou, 1998), and its cleavage leads to a 20-fold downregulation of Wee1 kinase activity. It is possible that Wee1 is a substrate for caspase-3 during myogenic differentiation. This is in agreement with the finding that Wee1 inhibition can restore myogenic fusion in QVD-treated cells. Why the disruption of G2/M checkpoint kinase
activity by caspase-3 would be a step in the program of myogenic differentiation is not immediately obvious.

The molecular mechanism through which Wee1 inhibition leads to DNA damage is still completely unknown. Likewise, the molecular mechanism through which Wee1 inhibition causes precocious differentiation is also still a mystery. In the next chapter, I will attempt to answer both of these questions.
Chapter 4

Destabilizing the G2/M checkpoint induces spontaneous myogenic fusion
Chapter 4: Destabilizing the G2/M checkpoint induces spontaneous myogenic fusion

4.1: Introduction

In the last chapter, it was shown that inhibition of Wee1 kinase induced spontaneous myogenic differentiation in myoblasts. This differentiation was accompanied with substantial nuclear DNA damage. The mechanism by which this DNA damage is induced is not immediately obvious. In this chapter, I attempt to develop a mechanistic explanation for how inhibition of Wee1 causes DNA damage and leads to myogenic differentiation.

Wee1 is primarily a cell cycle checkpoint kinase. Its function is to restrain cells at the G2/M checkpoint through inactivation of the CDK1/cyclin B complex. Wee1 phosphorylates CDK1 on tyrosine 15, rendering it inactive (Squire, 2005; Do, 2013). The function of the G2/M checkpoint is to prevent cells from entering mitosis with DNA damage. Wee1 is not the only kinase which has this function. Two other checkpoint kinases, ATR and Chk1, are also involved in controlling G2/M transition (Diagram 1) (Cliby, 1998; Zhao, 2002).
Replication protein A (RPA) coats single-stranded DNA lesions and recruits the DNA damage response protein ATR. ATR phosphorylates Chk1, which both inhibits cdc25 and activates wee1. Inhibitory phosphorylation of the phosphatase cdc25 prevents CDK1 activation and progression into mitosis. Wee1 kinase activity directly inhibits CDK1 through Y15 phosphorylation, leading to the same outcome. Together, this provides a ‘double lock’ mechanism to ensure that cell cycle progression is halted until DNA damage can be repaired.
As well as their role in G2/M progression, all three kinases also have roles in regulating the initiation of DNA replication, as well as regulating the rate of DNA synthesis to ensure that replication forks progress at a manageable speed (Anda, 2016; Syljuåsen, 2005; Beck, 2012). These checkpoint kinases also have a role in maintaining replication fork stability during DNA synthesis (Petermann, 2010; Friedel, 2008; Domínguez-Kelly, 2011). These roles in DNA replication may explain the rapid accumulation of DNA damage upon inhibition of Wee1.

In the context of myoblasts, one could imagine a scenario where inhibition of Wee1, Chk1 or ATR activity would cause uncontrolled replication fork firing throughout the genome. This would quickly deplete the cells nucleotide stocks and lead to fork slowdown and stalling, followed by collapse into double-stranded DNA breaks. It was shown in chapter 1 that induction of DNA strand breaks appears to be sufficient to induce myogenic differentiation. This could explain the spontaneous fusion seen under Wee1 inhibition in chapter 3. A model could be envisioned where disruptions in Wee1, Chk1 or ATR lead to uncontrolled replication initiation, nucleoside depletion, and replication fork collapse into strand breaks. Such strand breaks then induce the myogenic differentiation program. If this is truly the mechanism by which Wee1 inhibition leads to DNA damage, then inhibition of both Chk1 and ATR should mirror both the fusion effects and the DNA damage accumulation seen under Wee1 inhibition, and these effects should be blocked if replication initiation or strand breakage can be blocked.

In this chapter, I show that pharmacological inhibition of each of the checkpoint kinases; Wee1, Chk1 and ATR; induces spontaneous fusion of myoblasts, similar to that seen with PD-407824 treatment. Inhibition of each kinase is accompanied by the accumulation of a large number of
double-stranded DNA breaks. Along with this DNA damage, checkpoint kinase inhibition causes a rapid induction of DNA synthesis as measured by Brdu incorporation. This was accompanied by the appearance of mitotic figures containing substantial DNA damage, consistent with the roles of Wee1, Chk1 and ATR in maintaining the G2/M checkpoint.

The induction of DNA synthesis and accumulation of DNA strand breaks was blocked when cells were co-treated with cyclin-dependent kinase inhibitors, suggesting that the observed DNA damage was due, in part, to abnormal induction of DNA replication. Supplementation of cells with exogenous nucleosides also mitigated the appearance of DNA strand breaks, suggesting that replication fork collapse due to nucleoside depletion was the ultimate source of the DNA strand breaks.

Interestingly, depletion of CAD by siRNA was unable to block the spontaneous fusion seen under checkpoint kinase inhibition. This mirrors the finding in chapter 1, where UV-induced differentiation could not be blocked by CAD. This again suggests that DNA strand breaks in random locations are sufficient to induce myogenic differentiation.

Together, these findings suggest that the proliferating myoblast must retain tight control of DNA synthesis, as loss of genomic integrity can lead to spontaneous and abnormal differentiation. Moreover, this chapter provides evidence to a growing body of literature which suggests that checkpoint kinases which control the G2/M checkpoint also have distinct roles in controlling the onset and rate of DNA synthesis during S-phase.
4.2: Results

4.2.1: Pharmacological properties of checkpoint kinase inhibitors

For the purpose of this study, a panel of pharmacological inhibitors was used to target different cell cycle checkpoint kinases, similar to the methodology used in chapter 2. PD-407824 is an inhibitor of Wee1, but also has activity against Chk1. Because of this, a more selective and potent Wee1 inhibitor, MK-1775, was substituted for PD-407824, while Chir-124 was used as an inhibitor for Chk1. VE-821, the ATR inhibitor, was re-used from chapter 2. Although VE-821 showed no inhibition or enhancement of fusion compared to DNA-PK inhibition in chapter 2, only a single concentration was used. Given ATR’s role in the DNA damage response and G2/M checkpoint signaling, I thought it would be reasonable to try this drug over a wider concentration range.

Figure 1: Chemical structures of the checkpoint inhibitors used in this study.

Each of these compounds is potent inhibitor of its target kinase (Table 1). Chir-124 and VE-821 are both quite selective for their primary molecular targets. MK-1775 has somewhat less selectivity, as it has only 3-fold selectivity for Wee1 over Yes1, the cellular homolog of Yamaguchi sarcoma virus oncogene product (Hirai, 2009). However, MK-1775 has no detectable activity against either Chk1 or ATR.
Table 1: Chart of targets and cell free IC\textsubscript{50} values for each of the checkpoint inhibitors used in this study.

<table>
<thead>
<tr>
<th>Target</th>
<th>CHIR-124 IC\textsubscript{50} cell free (nM) (Tse, 2007)</th>
<th>MK-1775 IC\textsubscript{50} cell free (nM) (Hirai, 2009)</th>
<th>VE-821 IC\textsubscript{50} cell free (nM) (Reaper, 2011)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chk1</td>
<td>0.3</td>
<td></td>
<td>13</td>
</tr>
<tr>
<td>FLT3</td>
<td>5.3</td>
<td>14</td>
<td>&gt;1,000</td>
</tr>
<tr>
<td>PDGFR</td>
<td>6.6</td>
<td>&gt;500</td>
<td>2,200</td>
</tr>
<tr>
<td>GSK-3</td>
<td>23.3</td>
<td></td>
<td>3,900</td>
</tr>
</tbody>
</table>

4.2.2: Inhibition of ATR, Chk1, and Wee1 each induces spontaneous myogenic differentiation

To determine the effects of checkpoint kinase inhibition on myogenic differentiation, cells were treated with varying concentrations of MK-1775 (Wee1), Chir-124 (Chk1) and VE-821 (ATR). All three inhibitors induced spontaneous differentiation in cycling cultures maintained in growth medium (Figure 1A,B). Compared to cells induced to differentiate by serum withdrawal, kinase inhibition-induced fusion produced much larger myofibers with more nuclei within each fiber, suggesting that a greater number of individual fusion events occurred under kinase inhibition.

MK-1775 (Wee1) and VE-821 (ATR) showed a substantial increase in myogenic fusion at 1 nM (Figure 2). Interestingly, VE-821 showed no effect against fusion at the higher concentration of 100 nM (Chapter 2, Figure 4A). This suggests a hormesis-like dose response for this compound, in which a lower dose produces an effect which is absent at a higher dose (Calabrese, 2003). Chir-124 (Chk1) treatment in particular produces a marked effect on fusion at 100 pM, inducing the formation of large triangular sail-like myofibers which contain dozens of nuclei in their central region.
Figure 2: Checkpoint kinase inhibitors induce spontaneous myogenic fusion. Cells were treated with each of the three compounds and maintained in growth medium for 72 hours. Vehicle cells were induced to differentiate by serum withdrawal for 72 hours. Cells were then fixed and stained for myosin heavy chain. A: Checkpoint inhibitors produce abnormally large myofibers, similar to PD-407824 treatment. B: Effect on fusion index of each checkpoint kinase inhibitor. Concentrations used were: MK-1775, 1 nM; VE-821, 1 nM; Chir-124, 0.1 nM. Data represent means ± SEM of three independent experiments. Asterisks indicate significance at p < 0.05.

The data here support the assertion that the molecular target of PD-407824 is Wee1. Because Chk1 and ATR inhibition produce the same phenotype as Wee1 inhibition, these findings also suggest that the myogenic effect is caused through disruption of the G2/M cell cycle checkpoint, rather than through a peculiar activity of Wee1.
4.2.3: Checkpoint kinase inhibition causes rapid accumulation of DNA damage

In the last chapter, I showed that Wee1 inhibition accelerates the myogenic program by approximately 24 hours. I also showed that Wee1 inhibition caused accumulation of substantial DNA damage very rapidly after drug treatment. I interpreted this to indicate that accumulation of DNA damage itself directly triggers the myogenic gene transcription, essentially circumventing the earlier caspase \( \rightarrow \) CAD events. This is in line with the findings in chapter 1, which suggest that random DNA damage is sufficient to drive differentiation, and with the findings in chapter 2, which suggest that elements of the DNA damage response are required for the myogenic program to proceed.

If this DNA damage-induced differentiation model is correct, then the checkpoint kinase inhibitors should also produce DNA damage in cycling cultures before they begin cell fusion.

Myoblasts maintained in growth medium were treated with each of the three checkpoint kinase inhibitors at the concentration which produced the fusion effect described above. The cells were incubated for 6 hours, then fixed and stained for \( \gamma \text{H2AX} \). Each of the checkpoint kinase inhibitors caused substantial accumulation of \( \gamma \text{H2AX} \) (Figure 3A).

Interestingly, when cells are treated with each of the checkpoint kinase inhibitors, more mitotic figures are seen, and most of these mitotic figures also stain positive for \( \gamma \text{H2AX} \), indicating that these cells are proceeding through mitosis with substantial DNA damage (Figure 3B). This finding is consistent with the conventional roles of Wee1, Chk1 and ATR in maintaining the G2/M checkpoint. Destruction of this checkpoint causes cells to enter mitosis before DNA damage can
be repaired. This is also consistent with reports that inhibition of Wee1 leads to accelerated mitotic entry (Krajewska, 2013).
Figure 3: Checkpoint kinase inhibition produces DNA strand breaks. Myoblasts maintained in growth medium were treated with each compound for six hours, before being fixed and stained for $\gamma$H2AX. A: Checkpoint kinase inhibitors cause a substantial increase in $\gamma$H2AX staining (teal) compared to control. B: Checkpoint kinase inhibitors induce cells to enter mitosis with substantial DNA damage. Magenta is $\gamma$H2AX, green is tubulin, blue is Hoechst 33342. Inset: example mitotic figures showing cells at metaphase with DNA damage. A mitotic figure without DNA damage is shown inset in the ‘Vehicle’ panel. Data represent means ± SEM of three independent experiments. Asterisks indicate significance at p < 0.05.
Similar to PD-407824, all three checkpoint inhibitors rapidly induced DNA damage. The fact that all three inhibitors induced both spontaneous myogenic differentiation and substantial DNA damage suggests a common mechanism through which all three function. All three inhibitor targets; Wee1, Chk1 and ATR have roles not only in controlling cell cycle checkpoint progression, but also in DNA synthesis initiation. There is a growing body of evidence that G2/M checkpoint kinases also have roles in regulating DNA replication during normal S-phase (Garnier, 2009; Murga, 2009; Beck, 2012; Syljuåsen, 2005; Santamaría, 2007).
4.2.4: Checkpoint kinase inhibition causes a rapid increase in DNA synthesis through CDK dysregulation

Sørensen (2012) argue that Wee1, Chk1 and ATR not only control the G2/M transition, but also control replication initiation during normal S-phase. This system is essential to maintain genomic stability during normal cell replication (Diagram 2). Inhibition of these checkpoint kinases has been shown to induce unscheduled replication origin firing and rapid, uncontrolled DNA synthesis (Syljuåsen, 2005; Beck, 2012), as well as acceleration of mitotic entry. (Krajewska, 2013).

Diagram 2: Mechanism controlling replication initiation in cycling cells. Wee1 downregulates CDK1 activity through phosphorylation of the tyrosine 15 residue. Cdc25 releases this negative regulation by dephosphorylating tyrosine 15. ATR and Chk1 inhibit the phosphatase activity of Cdc25, tipping the balance in favor of replication suppression.
It has been shown that Wee1 inhibition leads to an increase in DNA synthesis concurrent with an increase in DNA damage (Beck, 2012). The authors argue that Wee1 has a role in controlling the rate of DNA synthesis, and thus is involved in maintaining genomic stability. A similar process may be able to explain the induction of DNA damage in myoblasts under checkpoint kinase inhibition. To test this, cells were co-treated with each of the checkpoint inhibitors and the synthetic thymidine analog bromodeoxyuridine (BrdU), whose incorporation into newly synthesized DNA can be detected with immunostaining. If checkpoint kinase inhibition causes dysregulated DNA synthesis, then there should be an increase in BrdU+ nuclei upon checkpoint kinase inhibition.

Treatment with MK-1775 (Wee1), Chir-124 (Chk1) and VE-821 (ATR) all caused a substantial increase in DNA synthesis by 3 hours post treatment (Figure 4A). This suggests that each inhibitor induced a burst of DNA synthesis relatively quickly and within the same timeframe in which DNA strand breakage occurs (Figure 3, above).

These results are consistent with a model in which checkpoint kinase inhibition causes dysregulated CDK activity and uncontrolled replication fork firing, leading to DNA strand breakage. However, it is also possible that the observed DNA synthesis is a consequence, not a cause, of DNA strand breaks. ‘Unscheduled DNA synthesis’ is a phenomenon which has been observed since the 1960s (Painter, 1967). This is where DNA synthesis occurs outside of the normal S phase of the cell cycle, and is indicative of DNA repair following genotoxic lesions. DNA strand break repair occurs through specialized detection and ligation mechanisms outlined in chapter 2. It could be argued that the increase in BrdU incorporation seen under checkpoint
kinase inhibition is due to a DNA repair process, rather than true DNA synthesis initiation through CDK/cyclin dysregulation.

To discriminate between these two possibilities, the same experiment described above was run in the presence of the CDK inhibitor purvalanol B. Purvalanol B has activity against several CDK/cyclin complexes (Diagram 3). If the increased DNA synthesis observed under checkpoint kinase inhibition is truly caused by CDK/cyclin-mediated replication origin firing, then purvalanol B should be able to block this effect. If the DNA synthesis is due to a DNA repair process, then purvalanol B should not be able to block Brdu accumulation.

Diagram 3: Structure and reported IC\textsubscript{50} values of purvalanol B against various CDK/cyclin complexes

Pretreatment with Purvalanol B strongly inhibits Brdu accumulation in checkpoint kinase-inhibited cells (Figure 4B). This appears to suggest that the observed DNA synthesis under Wee1, Chk1 and ATR inhibition was due to CDK-mediated replication origin firing, rather than a DNA repair process.
Figure 4: Inhibition of Wee1, Chk1 and ATR all induce abnormal DNA synthesis. Cells were pre-treated for 1 hour with or without the CDK inhibitor purvalanol B, and labelled with the synthetic nucleotide Brdu. Cells were then treated with each of the checkpoint kinase inhibitors. A: Each of the checkpoint kinases induced substantial incorporation of Brdu, indicative of *de novo* DNA synthesis. Teal is Brdu; blue is Hoechst 33342. B: Incorporation of Brdu can be blocked by the CDK inhibitor purvalanol B: indicating that the observed DNA synthesis was due to conventional DNA replication, rather than due to a DNA repair process. Asterisks indicate significance at p < 0.05.
In this section, it was shown that checkpoint kinase inhibition led to substantial induction of DNA synthesis. This is consistent with a model where dysregulation of Wee1, Chk1, and ATR all lead to aberrant activation of CDK/cyclins, and thus replication fork firing. It remains to be seen how this uncontrolled DNA synthesis leads to DNA strand breakage.

4.2.5: DNA damage is caused by unscheduled DNA replication initiation and replication fork collapse

It was shown above that inhibition of the checkpoint kinases Wee1, Chk1 and ATR all induced spontaneous differentiation in cycling myoblasts. This differentiation was accompanied with substantial DNA strand breakage and DNA synthesis. One model to explain these findings could be that inhibition of checkpoint kinases leads to dysregulation of CDK/cyclin activity. Dysregulation of CDK activity through inhibition of checkpoint kinases has been shown to cause uncontrolled DNA replication origin firing, leading to nucleotide exhaustion and collapse of replication forks into strand breaks (Anda, 2016; Syijuåsen, 2005; Beck, 2012). This mechanism explains the findings above that checkpoint kinase inhibition leads both to DNA synthesis as measured by BrdU incorporation and to DNA strand breaks as measured by γH2AX accumulation. If this CDK-mediated mechanism of DNA damage induction is correct, then some predictions can be made. In the above section, it was shown that inhibition of CDK/cyclin activity with purvalanol B inhibited uncontrolled DNA synthesis upon checkpoint kinase inhibition. Likewise, CDK/cyclin inhibition should block the formation of DNA strand breaks, as shown by Beck (2012). Additionally, if replication fork stalling and collapse is due to nucleoside depletion, then supplementing myoblasts with exogenous nucleosides should reduce strand break formation.
Cells were pretreated with either supplementary nucleosides or purvalanol B for one hour, then incubated with each of the checkpoint kinase inhibitors for 6 hours. Cells were then fixed and stained for $\gamma$H2AX. Each checkpoint inhibitor induced a considerable level of $\gamma$H2AX staining, consistent with DNA strand break accumulation (Figure 5). Treatment with supplementary nucleosides strongly reduced the formation of strand breaks, as did treatment with purvalanol B.
Figure 5: Checkpoint inhibition-induced DNA damage is caused by replication stress. A: Cycling myoblasts were pretreated for 1 hour with either supplementary nucleosides or with the CDK inhibitor purvalanol B (1 µM), then treated with each of the checkpoint kinase inhibitors for 6 hours. Cells were then fixed and stained for γH2AX. Both supplementary nucleosides and purvalanol B strongly inhibited the accumulation of γH2AX, suggesting that they prevented DNA strand break formation. Supplementary nucleosides were used at final concentrations of cytidine, 60 µM; guanosine, 60 µM; uridine, 60 µM; thymidine, 16 µM; and adenosine, 66 µM. Data represent means ± SEM of three independent experiments. Asterisks indicate significance at p < 0.05.
In this section, it was shown that the mechanism through which checkpoint kinase inhibition-induced strand breaks occur is likely due to CDK-mediated uncontrolled replication fork firing throughout the genome, followed by nucleotide exhaustion and replication fork collapse. Together with the above sections, these findings present a narrative in which checkpoint kinase inhibition induces aberrant DNA replication, followed by strand breakage, and that these strand breaks are responsible for the spontaneous fusion seen in figure 2.

4.2.6: Knockdown of CAD is unable to prevent checkpoint kinase inhibition-induced fusion

So far, it has been established that inhibition of the checkpoint kinases Wee1, Chk1 and ATR all caused spontaneous myogenic fusion, accompanied with substantial DNA strand breakage. It has also been shown that this DNA damage is accompanied by DNA synthesis as measured by BrdU incorporation, and can be inhibited by pre-treatment either with the CDK inhibitor purvalanol B or with supplementary nucleosides. This is consistent with a model in which checkpoint kinase inhibition causes unregulated DNA synthesis initiation, followed by replication fork collapse due to nucleoside exhaustion.

I hypothesize that this DNA damage is the source of the spontaneous myogenic fusion seen under checkpoint kinase inhibition, much like UV-induced DNA damage caused spontaneous differentiation in chapter 1. The implication of both findings is that DNA strand breaks in random locations throughout the genome are sufficient to trigger differentiation, rather than sequence-specific strand breaks. However, it is also possible that checkpoint inhibition leads to CAD activation, and thus to sequence-specific strand breakage, as suggested by Larsen (2010)
To rule out this possibility, the effect of CAD knockdown on checkpoint inhibition-induced fusion was assessed. If checkpoint inhibition induces fusion simply because it engages the CAD-directed DNA damage mechanism, then knockdown of CAD should block checkpoint inhibition-induced fusion. Conversely, if replication fork collapse-induced random DNA breaks are sufficient to induce differentiation, then CAD knockdown should have no effect upon checkpoint kinase inhibition-induced differentiation.

Cells were transfected with siRNA constructs against CAD. 24 hours post transfection, cells were treated with each of the checkpoint kinase inhibitors. Fusion was allowed to proceed for 72 hours, then the cells were fixed and stained for myosin heavy chain and the fusion index was enumerated. Treatment with the CAD siRNAs alone substantially reduced myogenic fusion (Figure 6), as seen before. Myoblasts treated with both checkpoint inhibitors and CAD siRNA constructs showed no difference in fusion compared to cells treated with checkpoint inhibitors and scramble siRNAs.
Figure 6: Knockdown of CAD by siRNA was unable to block checkpoint inhibition-induced myogenesis. Cells were first transfected with siRNA constructs targeting CAD. 24 hours after transfection, cells were washed once, then treated with each of the checkpoint kinase inhibitors. Fusion was allowed to proceed for 72 hours, then the cells were fixed and stained for myosin heavy chain. A basal level of fusion is seen in cells transfected with a scramble siRNA control. This is blocked in cells transfected with siRNA constructs targeting CAD (first row). Each of the checkpoint inhibitors produced an increase in myogenic fusion (first column), which was not blocked in CAD-knockdown cells. Data represent means ± SEM of three independent experiments. Asterisks indicate significance at $p < 0.05$. 
4.3: Discussion

In this chapter, it was shown that disruption of the key checkpoint kinases Wee1, Chk1 and ATR all induced spontaneous myogenic differentiation, as well as DNA strand breaks. It was determined that the source of these strand breaks was dysregulation of CDK/cyclin activity, leading to aberrant DNA synthesis initiation, nucleoside exhaustion, and replication fork collapse. These strand breaks induce the myogenic program, and this induction is independent of the canonical CAD pathway.

Based on the data presented in this thesis, a coherent model of myogenic differentiation is emerging in which DNA damage is used as a trigger to induce cell cycle withdrawal, and hence to begin the differentiation program. These findings appear to correlate with this in chapter 1, which suggest that random, non sequence specific DNA damage is sufficient to drive differentiation. DNA strand breaks produced by nucleoside depletion-induced replication fork collapse should not have any particular sequence specificity, and are certainly unlikely to target specific loci within the genome which must be ‘triggered’ to activate the myogenic program.

Although DNA damage accumulation explains the spontaneous fusion seen when cells are treated with checkpoint kinase inhibitors, this explanation does not explain the oversized, highly multinucleated myofibres which are observed under checkpoint inhibition (Figure 2). One potential explanation relies on the fact that myoblasts must be in G1 to differentiate (Okazaki & Holtzer 1966). Under checkpoint inhibition, cell accumulate large amounts of DNA damage, and because the G2/M checkpoint has been removed, these cells proceed through mitosis with this damage (probably accumulating more DNA damage in the process). Because the G1 checkpoint is still functional, the cells are immediately arrested as soon as they exit mitosis. From G1 arrest,
they withdraw from the cell cycle and differentiate (Diagram 4). This could represent a general explanation for how DNA damage induces myogenic differentiation, although it does not explain the requirement for a functioning DNA damage response (Chapter 2).
Diagram 4: Proposed mechanism of DNA strand break induction in checkpoint kinase-inhibited myoblasts. 1: Treatment of cells with checkpoint kinase inhibitors induces uncontrolled DNA synthesis through dysregulated CDK/cyclin activity. 2: As a consequence of nucleotide depletion, replication forks stall and collapse into strand breaks. 3: Cells bypass the G2/M checkpoint and proceed through mitosis with substantial DNA damage. 4: Emerging from mitosis, cells are immediately arrested at the still functional G1 checkpoint, which induces cell cycle withdrawal and myogenic differentiation. Because checkpoint kinase inhibition accelerates mitotic entry and the G2/M checkpoint has been disrupted, and because the G1 checkpoint is still functional, more cells are halted in G1 under checkpoint kinase inhibition than under normal serum withdrawal. Since cells must be in G1 to differentiate, this means the pool of fusable cells is larger under checkpoint differentiation. A larger pool of fusible cells means more fusion events will take place, explaining the oversized myotubes seen under Wee1, Chk1 and ATR inhibition.
Interestingly, each of these checkpoint kinases appears to be downregulated during myogenic differentiation. Wee1 is downregulated during myogenesis at both the transcriptional and protein level (Sarkar, 2010; Shen, 2003). The findings here suggest that Wee1 downregulation is involved has a functional role in the myogenic program, since pharmacological downregulation of Wee1 induces spontaneous differentiation. Chk1 levels are downregulated over the course of myogenic fusion, and appears to be completely absent in terminally-differentiated myotubes (Fortini, 2012) and Chk1 has been shown to suppress myogenic differentiation through phospho-inhibition of p21 (Ullah, 2011). Together, this suggests that, like Wee1, Chk1 downregulation has a functional role in myogenesis. Forced expression of an extra copy of the ATR locus from 3q inhibits the function of the myogenic transcription factor MyoD, and strongly inhibits myogenic differentiation (Smith, 1998). This is in accord with the data presented here which shows that inhibition of ATR causes prolific myogenic differentiation. Together, these finding may suggest that downregulation or inhibition of ATR is a necessary step in myogenic differentiation.

Wee1, Chk1 and ATR are all required for life, as knockout organisms of each display early embryonic lethality (Brown, 2000; Takai, 2000; Tominaga, 2006). These checkpoint kinases are also considered targets in cancer chemotherapy. Currently, there are compounds in phase I and II clinical trials targeting each of these targets (AstraZeneca, 2014; National Cancer Institute, 2012; National Cancer Institute, 2014). Given the effect of checkpoint kinase inhibitors on myogenesis described in this chapter, it is possible that such a clinical strategy could have undesirable effects on muscle regeneration. Since inhibition of these checkpoint kinases causes spontaneous differentiation of myoblasts, it is possible that these drugs could cause premature depletion of the pool of myogenic precursor cells, of which there is only a limited supply in adults. This could
strongly hamper the ability of muscle to regenerate in response to cancer-induced cachexia and weightloss.

In this chapter, a further example of DNA damage-induced myogenic differentiation has been shown. These findings are in accord with those in chapter 1, which showed that UV-induced random DNA damage was sufficient to drive differentiation. They also accord with the findings in chapter 2 which suggest that a functioning DNA damage response is required for myogenesis to proceed. This data also bolsters findings in the literature which suggest that G2/M checkpoint kinases have separate roles in regulating S-phase DNA replication and maintaining genomic stability.
General Discussion
General Discussion

Programmed DNA damage: current state of the field

Evidence for programmed DNA damage in other systems

DNA damage- particularly double stranded DNA breaks- is typically seen as a very negative occurrence which must be resolved as soon as possible, lest they give rise to mutations or chromosomal breaks. In light of this, the idea of a programmed form of DNA damage which is deliberately induced as part of a normal cellular process may be hard to accept. In spite of this, there are several cellular processes in which programmed DNA damage is accepted and uncontroversial.

One example is V(D)J recombination, vital to generating immunoglobulin and T-cell receptor diversity in B-cells and T-cells respectively. In V(D)J recombination, Recombination Activating Genes (RAGs) induce DNA strand breaks between different coding segments. This facilitates segment shuffling in order to produce novel combinations of variable, diversity, and joining segments. Such transient strand breaks are tolerable because they are quickly bound and resected by the non-homologous end-joining DNA repair mechanism (Nussenzweig, 1996; Ma, 2002). V(D)J recombination represents a mechanism in which the cell generates semi-random double-stranded breaks as part of a defined program. Interestingly, it has been shown that RAG-induced strand breaks have a wider function in B-cell maturation. These strand breaks not only contribute to immunoglobulin gene segment recombination, but also lead to the induction of an entire program of gene expression that facilitate many processes required for differentiation such as cell homing and migration (Bredemeyer, 2008).
Another uncontroversial example of programmed DNA damage is found in meiotic recombination. During meiosis, homologous chromosomes are paired and corresponding sequences are swapped between chromosomes. This is done to facilitate genetic diversity in offspring. In this process, double-strand breaks are induced by Spo11. Recombinases RAD51/DMC1 then facilitate strand invasion and sequence exchange (Keeney, 2006). This recombination process also appears to be semi-random. Corresponding sequences are normally swapped from equivalent loci on sister chromatids, however the actual loci which are selected for recombination cannot be predicted in advance.

Although the above are uncontroversial examples of programmed DNA damage, the concept of programmed DNA damage as part of a cellular terminal differentiation process is a much less widely accepted idea. However, there are several examples which can be drawn upon.

In several cell types, it has been discovered that terminal differentiation programs can be induced by exposure to exogenous sources of DNA damage, such as ionizing radiation or genotoxic drugs. Melanocyte stem cells, which mature into melanin-producing cells, have been shown to undergo terminal differentiation when exposed to DNA-damaging ionizing irradiation (Inomata, 2009). Interestingly, this could represent a mechanism through which skin tanning and freckle formation are regulated by sunlight exposure. Likewise, ionizing irradiation induces the differentiation of hematopoietic stem cells through the activity of the DNA damage response protein Gadd45a (Wingert, 2016). As another example, primary myelogenous cells can be induced to differentiate by treatment with the DNA damaging agent doxycycline (Santos, 2014). It is unclear whether this differentiation is simply a stress-response to an exogenous stimulus, or whether the exogenous
DNA damage mimics a physiological DNA damage program. None of these differentiation programs, however, has yet shown a physiological DNA fragmentation process comparable to the caspase-3 > CAD axis identified in myoblast differentiation.

More in line with the theory of a programmed DNA damage, there are some examples of cell differentiation in which DNA damage seem to occur as part of the differentiation program. Promyeloid HL-60 cells have been shown to accumulate DNA strand breaks during their differentiation program (Farzaneh, 1987) and treating this cell line with the DNA-damaging agent genistein induces spontaneous myeloid differentiation (Constantinou, 1990). Likewise, granulocytic differentiation of monocytes has been shown to be accompanied by the induction of DNA strand breaks (Khan, 1987), and that this is due to the action of the differentiation-specific Macrophage and Granulocyte Inducer type 2 (MGI-2) (Lord, 1988). In developing spermatocytes, transient DNA strand breaks occur within the chromatin, and these breaks are later repaired (Joshi, 1990; Iseki, 1986; Marcon, 2004). Finally, erythroblasts accumulate DNA strand breaks during differentiation, and these cells can also be artificially induced to differentiate by exogenous DNA damaging agents including X-irradiation and bleomycin treatment (Scher & Friend, 1978). Interestingly, the process of erythroblast differentiation has been shown to rely on a non-apoptotic caspase-3 activity (Zermati, 2001; Carlile, 2004), similar to myogenic differentiation.

Evidence for programmed DNA damage in myogenesis

Farzaneh (1982) demonstrated that differentiating cultures of myoblasts accumulate DNA strand breaks. Several years later, it was demonstrated that this DNA damage is repaired over the course of the myogenic differentiation program (Dawson & Lough, 1988), and that conventional DNA
damage response pathways are active at this time (Jost & Jost, 1994). Shiokawa (2002) showed that a nuclease activity is responsible for generating these myogenic strand breaks, and Larsen (2010) revealed that the nuclease to be Caspase-Activated DNase (CAD), and that this was activated as the result of an apparently non-apoptotic function of caspase-3 (Fernando, 2002).

Investigations into the nature of this DNA damage have produced conflicting and contradictory results. Some have argued that DNA damage itself actually prevents myogenic differentiation and that if substantial DNA lesions are detected, the differentiation program is brought to a halt until the DNA damage is resolved. Others have put forward evidence for the opposite view; that DNA damage can actually trigger myogenic fusion.

Puri (2002) proposed a myogenic DNA damage checkpoint, wherein a myoblast undergoing differentiation will halt its differentiation program if substantial DNA damage is detected. The explanation put forward is that the myoblast must ensure any existing DNA lesions are repaired before it commits to a postmitotic state within a differentiated myotube (Vahidi Ferdousi, 2014; Narciso, 2007).

Mechanistically, the experimenters used three genotoxic DNA damaging agents (etoposide, cisplatin and MMS) to induce strand breaks, and then measured the ability of myoblasts to undergo differentiation. They showed that a reversible block in myogenic differentiation can be induced by these agents, and that withdrawal of the drugs can restore differentiation. Thus Puri et al suggest that DNA damage is profoundly anti-differentiation signal within the cell, forcing cells to halt differentiation until DNA can be repaired.
However, a significant issue with this experiment is that each of these drugs on its own has effects on DNA synthesis, gene transcription, and cell cycle progression which can explain these observations. Etoposide blocks not only DNA synthesis but also prevents gene transcription (Korwek, 2012). Since myogenesis requires the coordinated activation of an entire program of genes, etoposide’s inhibitory effect can probably be explained by its repression of gene transcription. Cisplatin induces cell cycle arrest in G2 (Sorenson, 1990) and MMS blocks S-phase progression (Shirahige, 1998). Myogenic cell cycle withdrawal occurs at G1 (Okazaki & Holtzer 1966), suggesting that these agents simply prevent myoblasts from progressing through S/G2 and reaching G1.

Others have argued the opposite position, that DNA damage itself can trigger myogenic differentiation. Hossain (2003) demonstrated that treatment of Drosophila Schneider myoblasts with the DSB-inducing agent neocarzinostatin causes them to exit from the cell cycle, express myosin heavy chain, and fuse into multinucleated myotubes, all the features of normal differentiation. No induction of differentiation was seen when cells were treated with agents which inhibited proliferation, indicating that DNA damage itself is the causal agent of differentiation, rather than a perturbation of the cell cycle.

Open questions in the field

1. Is DNA damage sufficient to induce myogenesis in mammalian myoblasts?

Hossain (2003) demonstrated that Drosophila myoblasts can be induced to differentiate by exposure to DNA damaging drugs. Puri (2002) showed that C2C12 myoblast differentiation can be blocked by exposure to genotoxic drugs. These two findings appear to be in contradiction.
Larsen (2010) showed that CAD-induced strand breakage is required for myogenic differentiation. An open question is whether DNA damage on its own is sufficient to induce myogenic differentiation, or if there are other factors which are also required.

2. Is myogenic DNA damage sequence-specific or non-specific?
Larsen (2010) showed that an upstream site within the promoter region of $p21^{Waf1}$ is selectively cleaved during myogenic differentiation, and that when CAD is knocked down, this cleavage is reduced. No such cleavage was found for other genes tested. Although this is in accordance with a sequence-specific model, it does not convincingly prove this model. It still remains to be seen whether CAD activity is localized to particular, defined sites; or distributed randomly throughout the genome.

3. How does myogenic DNA damage contribute to the differentiation program?
CAD induces strand breakage during myogenesis, and knockdown of CAD blocks both strand breakage and the myogenic program (Larsen, 2010). If DNA damage is induced as part of the myogenic program, what is its function? It has been shown that during myogenesis, there occurs not just DNA damage, but also DNA repair (Farzaneh, 1985; Dawson & Lough, 1988; Jost & Jost, 1994; al-Khalaf, 2016; Connolly, 2016). It is possible that elements of this repair mechanism are involved in the progression of the myogenic program, perhaps controlling gene expression through transcription factor activation.
Major findings of this thesis

*Random DNA damage is sufficient to induce myogenic differentiation*

Two different models could be proposed to explain the nature of CAD-induced DNA damage (Diagram 1). Larsen (2010) propose a proximity model in which CAD-induced DNA damage occurs in sites proximal to myogenic genes, leading to their activation. This model is problematic because CAD has no particular sequence specificity, and because no epigenetic mechanism has been uncovered to explain how only certain sequences such as myogenic promoter elements are exposed to CAD cleavage.

It is also possible to conceive of a different model in which CAD-induced strand breaks are distributed in an essentially random manner. In this model, the myogenic effect is due to elements of the DNA damage response activating myogenic transcription factors such as MyoD and myogenin. In this model, it is irrelevant which sites are targeted *per se*, rather the magnitude of the DNA damage response generated is the main driver of the myogenic program.
Diagram 1: Proximity vs random models of myogenic DNA damage. In the proximity model (A), strand breaks are enriched at sites proximal to myogenic open reading frames. In the stochastic model (B) strand breaks are randomly distributed throughout the CAD-accessible chromatin.

The primary implication of the second theory is that random DNA damage from an exogenous source should be able to trigger myogenic differentiation. This should not be the case with the sequence specific model. The stochastic model poses a risk that sequences within important reading frames could be cleaved. Diagram 1 is not to scale, however. Protein coding sequences comprise less that 2% of the human genome (Lander, 2001; D'Angelo, 2011), and only a fraction of these are relevant for the process of myogenic differentiation or for the metabolic activity of postmitotic myotubes.

In chapter 1, it was shown that irradiation of myoblasts with UV light will trigger their differentiation. This on its own suggests that random DNA damage is capable of inducing myogenic differentiation, however it could still be argued that UV irradiation induces CAD
activity, and this CAD activity produces sequence-specific strand breaks, in line with the ‘proximity’ model. However, was also shown that this effect occurs even when CAD is knocked down by siRNA. It was also shown that UV irradiation was able to partially rescue Q-VD-OPh-inhibited differentiation, suggesting that some aspects of myogenic caspase activity can be supplied by random irradiation. Taken together, the data in chapter 1 are in accord with a model where random, non-sequence specific DNA damage is sufficient to trigger differentiation.

Likewise, in chapters 3 and 4, it was shown that treatment of myoblasts with checkpoint kinase inhibitors caused substantial DNA damage, followed by myogenic differentiation. The extent of this DNA damage is similar to that seen during serum withdrawal-induced differentiation. This DNA damage is accompanied by Brdu incorporation, indicative of DNA synthesis. The accumulation of strand breaks can be blocked by pre-treatment of cells with CDK inhibitors or supplementary nucleotides. Taken together, this suggests that checkpoint kinase inhibition-induced DNA damage is produced as the result of unscheduled replication origin firing throughout the genome, which leads to nucleotide exhaustion, replication fork slowdown, and finally collapse into strand breaks. Checkpoint kinase inhibition-induced fusion was able to proceed even when CAD was knocked down by siRNA. Similar to the data presented in chapter 1, this suggests that DNA damage produced from an exogenous source was able to induce myogenic fusion.

The data presented here cannot rule out a semi sequence specific model, in which DNA strand breakage is random, but the sequences available for cleavage are restricted through an epigenetic mechanism. Histone methylation could represent one such mechanism. Interestingly, one of the hit compounds from the kinase inhibitor compound screen presented in chapter 3 was ryuvidine,
an inhibitor of SETD8 methyltransferase. This enzyme is responsible for methylating the lysine 20 tail of histone H4 (Jørgensen, 2013). This results in local chromatin condensation (Nishioka, 2002), and the absence of lysine 20 methylation leads to loosening of the chromatin structure (Oda, 2009). The fact that treatment with the SETD8 inhibitor ryuvidine strongly inhibits myogenic differentiation may indicate an important role for chromatin structure during differentiation, in line with the semi sequence-specific model. Disruptions of the chromatin structure could lead to the exposure of more sequences to CAD than are normally available. This would cause much greater strand breakage that is called for during the myogenic program.

Taken together, the weight of evidence presented here suggests that random DNA damage is sufficient to induce myogenesis. Whether myogenic strand breaks are truly distributed randomly, or whether they are distributed in a semi-random fashion controlled by epigenetic mechanisms will be a subject of future work.

**CAD is dispensable for myogenesis if exogenous DNA damage is supplied**

Myogenic differentiation relies on the activity of apoptotic caspases. One mechanism through which caspases facilitate myogenesis is through activation of CAD. CAD is an endonuclease which is activated through cleavage of its inhibitory partner ICAD by caspase-3. Once unleashed, CAD cleaves internucleosomal DNA. In chapter 1, it was shown that exposure of myoblasts to UV irradiation was able to induce myogenic fusion. Furthermore, it was shown that this myogenic effect occurs even if CAD is depleted by siRNA. These results were mirrored in chapter 4, where it was shown that checkpoint kinase inhibition induced substantial DNA damage as well as
spontaneous myogenic fusion; and that this effect could proceed even if CAD was inhibited by siRNA.

Together, these findings suggest that CAD is the physiological mechanism through which myogenic DNA damage is formed, but that CAD activity can be circumvented if exogenous sources of DNA damage are supplied. This finding may be of significance, because it suggests that exogenous DNA damage can trigger the premature differentiation of myoblasts. Myoblasts exist in a quiescent state within the basal lamina of muscle fibers. Upon injury, these cells are activated, proliferate, and undergo terminal differentiation. If exogenous DNA damage is capable of directly inducing myoblast differentiation, exposure to such damage could disrupt the quiescent state of myoblasts. Cells are constantly exposed to internal and external sources of DNA damage such as reactive metabolites and oxygen species, mistakes in DNA replication, and UV from sunlight exposure. Cells are also be exposed to sources of DNA damage from both medications such as chemotherapeutics (Cheung-Ong, 2013) and from illicit street drugs such as opiates (Shafer, 1994), as well as from environmental pollutants (Huang, 2012).

All of these sources of genotoxic lesions could potentially trigger the premature differentiation of myogenic cells. Given the fact that adults have only a limited supply of such myogenic cells, long term exposure to such agents could lead to exhaustion the myogenic pool. This in turn would strongly hamper the ability of the muscular system to repair and regenerate itself. Indeed, in humans there is a strong increase in the accumulation of oxidative DNA damage in skeletal muscle with increased age (Mecocci, 1999) and this is accompanied by a strong decrease in the number of myogenic satellite cells available for regeneration (Renault, 2002). This is in line with the
predominant ‘DNA damage theory’ of aging (Freitas, 2001), which posits that the major cause physiological aging in higher organisms is the progressive accumulation of DNA damage, and that this manifests as the gradual loss functional and regenerative capacity in tissues and organs.

A stoichiometric model of DNA damage-induced differentiation

Cells continuously accumulate DNA damage. It has been estimated that each mammalian somatic cell accumulates as many as 50,000 single stranded breaks per day and 50 double stranded breaks per day (Villenchik & Knudson, 2003; Tice & Setlow, 1985). Such DNA lesions are usually rapidly repaired, and have little consequence for the cell. If exogenous DNA damage is sufficient to induce myogenic differentiation, then the question arises; why do these natural and persistent sources of DNA damage not trigger spontaneous differentiation of quiescent myoblasts?

In chapter 1, it was shown that supplying exogenous DNA damage was sufficient to induce myogenic differentiation. This effect is dose-dependent, since irradiation for 15 seconds produces a low level of spontaneous fusion, while irradiation for 30-60 seconds produces a peak of myogenesis, and irradiation for longer times produces cell death. This suggests that there is a threshold effect, below which the cell can sustain DNA damage without induction of terminal differentiation. On the other hand, induction of high levels of DNA damage through longer exposure times causes substantial necrotic cell death. In between, there appears to be a level of UV-induced DNA damage which can induce myogenic differentiation. The same threshold effect was also observed when cells were treated with the wee1 inhibitor PD-407824. Treatment with 0.1 µM PD4 had no effect upon differentiation. Treatment with 0.3-1 µM induced a substantial increase in differentiation. At concentrations higher than 1 µM, cell death was induced.
Together, this suggests that DNA damage from exogenous sources such as drugs or UV can induce myogenesis, but only up to a point. In the case of PD4, the very sudden onset of cell death between 1 µM and 3 µM suggests that the level of DNA damage which is able to induce myogenesis is rather close to that which will induce catastrophic cell death.

*A functioning DNA damage response mediated by DNA-PK is required for differentiation*

Farzaneh (1985) and Dawson & Lough (1988) demonstrated that DNA strand breaks during myogenic differentiation are transient in nature. Jost & Jost (1994) showed that base excision repair is involved in the differentiation process. An open question is whether this DNA repair process is required for myogenic differentiation, or is just a consequence of DNA damage. That is, does the DNA damage response have a functional role in the myogenic program?

In chapter 2, it was shown that inhibition of the PIKK kinase DNA-PK blocks the phosphorylation of histone H2AX which occurs during myogenic differentiation as a consequence of CAD-mediated strand breaks. This occurs without inhibition of DNA damage induction, or of caspase-3 activity, suggesting that blocking DNA-PK activity genuinely prevents this DNA damage response. Inhibition of DNA-PK also blocks myogenic fusion, suggesting that the activity of DNA-PK is required for differentiation. A recent report suggests that another DNA damage response kinase, XRCC1, is also required for myogenic differentiation (al-Khalaf, 2016). Interestingly, DNA-PK has been shown to recruit XRCC1 in response to double-stranded breaks produced by ionizing irradiation (Lévy, 2006) and in response to unresencted stalled replication forks (Ying,
Together, these findings suggest that the myogenic DNA damage response has an indispensable functional role in myogenesis.

What lies downstream of the DNA damage response is still unknown. One could speculate that gene transcription is modified through the activity of DNA damage response kinases on myogenic transcription factors. This theory will be tested in future work (see next section).

Caspase-3 has more than one role in myogenesis

Apoptotic caspases can be divided into two classes, upstream ‘activator’ caspases and downstream ‘killer’ caspases. Killer caspases are so called because they are the effectors of apoptotic cell death, responsible for systematically dismantling and breaking down different components of the cell. It was long thought that killer caspases had only one function, as the executioners of apoptosis. This was a reasonable assumption, because caspases are proteases with thousands of substrates within the cell, and have the ability to autoactivate. It was difficult to conceive of a situation in which caspase activity could be unleashed within the cell without leading to apoptotic cell death.

Despite this, a substantial body of literature describing non-apoptotic roles of caspases has emerged in recent years (reviewed in Connolly, 2014). Caspases have been shown to be involved in a wide range of cellular processes, from cell differentiation to cell migration to intercellular communication. It is now possible to think of effector caspases as not just killers, but as cellular self-modification enzymes, used to carry out cell fate choices involving large-scale physical transformation of the cell, and that apoptosis is just a particularly extreme example of such a cell fate choice (Fernando, 2007).
The requirement for caspase activity in myogenic differentiation was first demonstrated in 2002 (Fernando). Since then, there have been attempts to elucidate the exact nature of this role. Caspases have been shown to have a wide range of functions in many different cell types, so caspases could have any number of functions in myogenesis. Based on earlier evidence showing that apoptotic-like strand breaks occur in differentiating myoblasts (Farzaneh, 1982; Coulton, 1992; Shiokawa, 2002), and it was shown that the source of these strand breaks was the activity of CAD (Larsen, 2010).

Myogenic fusion can be restored when CAD activity is blocked by siRNA if an exogenous source of DNA damage is supplied (Chapter 1, 3, 4). However, myogenic fusion cannot be restored by exogenous DNA damage if caspases themselves are pharmacologically inhibited. Taken together, these two findings suggest that caspases have more roles in myogenic differentiation than supplying DNA damage through CAD activity. If the only role of caspase activity was to supply DNA damage, then exogenous DNA damage should be able to restore QVD-inhibited fusion just as it is able to restore siCAD-inhibited fusion. There is at least one example of an apparently non DNA damage-associated role of caspases during myogenesis. Cleavage activation of Mammalian Sterile Twenty-like kinase (MST1) is a required event in myogenic differentiation (Fernando, 2002). The authors suggest that this MST1 activity via the p38 MAPK pathway, a stress response mechanism.

In other systems, effector caspases have been shown to be involved in cell migration (Gdynia, 2007), cytoskeletal reorganization (Mashima, 1999; Westphal, 2010), plasma membrane inversion to expose phosphatidylserine (Segawa, 2014), generation of extracellular signaling factors
(Lauber, 2003) and membrane blebbing which leads to the formation of lipid vesicles which can also function as signaling elements (Hristov, 2004). All of these processes are also key to myogenic differentiation. Cell motility is key for myoblasts to find and align with fusion partners (Vasyutina, 2006; Rochlin, 2010). The cytoskeleton is reorganized in differentiating myoblasts to facilitate fusion into multinucleated synctyia (Costa, 2014). Phosphatidylserine is exposed on the extracellular surface at the point of contact between myoblasts which are committed to fuse (van den Eijnde, 2001; Jeong, 2011). Caspase-generated extracellular signaling factors such as prostaglandin E2 and sphingosine 1-phosphate have been shown to facilitate myoblast proliferation and differentiation (Mo, 2015; Beaulieu, 2012; Squecco, 2006), and committed myoblasts secrete and exchange membrane-bound vesicles prior to cell fusion (Doberstein, 1997). Each of these represents a potential novel non-apoptotic role for killer caspases, and warrants further investigation.

Caspase-3 activity: cell autonomous vs apoptotic signaling

There is an ongoing controversy regarding the role of caspase activity in myogenic differentiation. One interpretation is that this is strictly an apoptotic effect. In this interpretation, apoptotic cells themselves are the source of myogenic signals. This is a version of the ‘phoenix rising’ compensatory proliferation phenomenon. When cells are induced to differentiate, a certain fraction of the cells die by apoptosis, accompanied by caspase activity. These apoptotic cells produce signaling factors which

This is an attractive model from a physiological viewpoint. Skeletal muscle is made up of bundles of fibers composed of elongated multinucleate cells. Quiescent myoblasts are distributed along the
length of these fibers, within the basal lamina. Injuries to skeletal muscle manifest as tears within of individual muscle fibers. Cells at the “break-point” die by necrosis, but it has also been shown that shortly after injury, cells at the periphery of the new break point die by a wave apoptotic cell death. An open question is: how are quiescent myoblasts “awakened” and induced to proliferate and migrate to the site of injury? Pro-proliferative and chemotactic signals emanating from apoptotic myonuclei at the site of injury present an elegant mechanism to explain this.

The most convincing experiment to date for the apoptotic signaling interpretation was performed by Hochreiter-Hufford (2013). In this experiment, it was shown that co-incubation of cycling myoblasts with apoptotic myoblasts was able to induce terminal differentiation, even when caspase activity was suppressed with pharmacological inhibitors. It was then shown that the source of the myogenic effect was due to direct cell-cell interaction between the apoptotic and cyclin cells. Phosphatidylserine exposed on the surface of the apoptotic cell binds to the BAII receptor on the cycling myoblast, and this leads to an intracellular signaling cascade which induces terminal differentiation. On the face of it, this seems like convincing evidence for the apoptotic signaling theory, however one problem with this approach is that it has been shown that differentiating myoblasts expose phosphatidylserine as part of their differentiating program (van den Eijnde, 2001). This happens at the sites of membrane and likely facilitates cell-cell recognition and anchorage. It has also been shown that artificial phosphatidylserine vesicles can also induce terminal differentiation (Jeong & Conboy, 2011).
The other school of interpretation suggests that caspase activity has a true non-apoptotic role in myogenesis.

In this thesis, it was shown that myoblasts accumulate DNA damage during myogenic differentiation, that this DNA damage is the result of caspase-3 activity, and that differentiation can be directly induced by exogenous sources. On its own, this could be used as evidence for either the cell autonomous or the apoptotic signaling model. One interpretation is that this represents a programmed DNA damage which occurs downstream of a cell autonomous caspase activity. On the other hand, it is entirely possible that the observed caspase-dependent DNA damage is coming from a fraction of the cells which are undergoing apoptotic cell death. In this scenario, the CAD-induced DNA damage is simply indicative of apoptotic cells, and that these cells are inducing myogenic differentiation through apoptotic signaling.

In the literature cited in this thesis, the involvement of DNA damage has generally been thought of as a cell autonomous event which occurs as part of the myogenic program. There are several justifications for this: 1. the DNA damage is usually seen in morphologically normal cells which do not bear gross feature of apoptosis; 2. the cells in which caspase activity and DNA damage is seen are generally found either localized within the basal lamina of the muscle fiber, or central within the regenerating fiber, two locations with which regenerative myoblasts are associated; 3. the DNA damage observed is generally ‘milder’ than that seen during apoptotic cell death, with only partial laddering of chromatin and less intense γH2AX staining. Although these are disparate findings collected from many separate experiments in *in vitro* and *in vivo* systems, together they seem to tip the balance in favor of a cell-autonomous DNA damage model. If the source of
myogenic DNA damage is caspase activity, then this appears to suggest that myogenic caspase activity is cell autonomous as well.

Another piece of evidence in favor of the cell autonomous model is the occurrence of DNA repair. It has been shown that not only do DNA strand breaks occur within cultures of differentiating myoblasts, but these breaks are also repaired over time. This has been shown not just through the disappearance of DNA damage (Farzaneh, 1982; Dawson & Lough, 1988; Larsen; 2010), but also through the active involvement of DNA repair pathways (Jost & Jost, 1994; Al-Khalaf, 2016). This is bolstered by the findings in chapter 2 and in (Connolly, 2016) which show that the DNA damage response kinase DNA-PK is required for myogenic differentiation. Apoptotic cells do not repair their DNA. The theory that myogenic DNA damage is localized within apoptotic cells cannot account for this requirement for DNA repair.

In summary, the data presented here, as well as that in the literature, suggest that myogenic DNA damage is more likely to be cell autonomous. If caspase-3 activity is the true source of myogenic DNA damage (and experiments with CAD knockdown suggests that it is), then this implies that myogenic caspase activity is also cell autonomous.

Future work

Events downstream of DNA damage: DDR-induced gene expression?

The data in this thesis suggest that DNA damage is required for myogenic differentiation. The source of this DNA damage is caspase activity, through the action of CAD. The most obvious question that arises from this work is: what is the function of this DNA damage? Double-stranded
DNA lesions are extremely dangerous for the cell, as they can lead to mutations and chromosomal rearrangements. Why do cells deliberately chop up their own DNA, only to immediately put it back together? Because of the risk associated with this activity, it is unlikely that this is a mere side effect of caspase activity. It likely has an active role in the myogenic program.

It was shown in chapter 2 that elements of the DNA damage response are required for myogenic differentiation, in this case the non-homologous end joining kinase DNA-PK. This is mirrored by the recent finding that another DNA damage response kinase, XRCC1, is also associated with myogenic differentiation (Al-Khalaf, 2016). Together, this suggests a model where DNA damage response kinases are responsible for carrying out some downstream function in the myogenic program.

One possible explanation is that these DNA damage response kinases are used to activate the myogenic gene program. DNA damage response kinases tend to be quite prolific, phosphorylating a wide range of substrates. This is consistent with their key role in making cell fate choices in response to DNA damage. When DNA damage is sensed, cells may decide to repair the damage, to halt the cell cycle and become quiescent, or to engage in programmed cell death. Perhaps cell differentiation is another cell fate choice that can be made in response to DNA damage. The myogenic gene program is organized in a tree-like structure, where a small number of apical transcription factors activate hundreds of downstream genes. It is possible that the myogenic DNA damage response is involved in activating these apical transcription factors.
Interestingly, there is some evidence that DNA damage response kinases interact with myogenic transcription factors. It has been shown that the trimeric Ku/DNA-PKcs complex can activate the androgen receptor, a major myogenic transcriptional regulator (Mayeur, 2005; Vlahopoulos, 2005). This activation can apparently proceed even in the absence of ligand. This could represent one mechanism through which DNA-PK engages the myogenic program, and warrants further investigation.

Another future avenue of work involves combining the findings of chapter 1 and chapter 2. If the myogenic gene program is engaged through DNA damage response factors, then it stands to reason that inhibition of DNA-PK should be able to block UV-induced differentiation.

*The number of DNA strand breaks sufficient to induce myogenesis*

The main thrust of this thesis was to investigate the occurrence and role of DNA strand breaks during myogenic differentiation. It has been shown in the literature that DNA strand breaks occur during differentiation, and that these are generated as a caspase-dependent process. DNA strand breaks are clearly lethal to the cell in high numbers. On the other hand, there is a basal level of strand breaks which occur in normal cycling cells as a result of routine physiological stresses such as oxygen radical exposure or mistakes in DNA replication. Such a low level of DNA damage is clearly tolerable for myoblasts, since cycling myoblasts do not spontaneously differentiate. It stands to reason that the level of DNA damage which is capable of inducing myogenic differentiation lies somewhere between these two extremes.
One avenue for future work will be to determine the number of strand breaks which are sufficient to induce myogenesis through UV exposure, and compare this to the number of strand breaks which occur during canonical myogenesis. UV irradiation allows for the precise control of the level of DNA damage by varying the exposure time, so the range of DNA strand breakage which is sufficient to induce myogenesis can be mapped with good accuracy. In early studies of the DNA damage during myogenesis, it was shown that differentiating chick myoblasts accumulate approximately 300 DNA strand breaks per nucleus (Fazlaneh, 1982). This estimate was produced using alkaline sedimentation of radiolabelled DNA strands isolated from differentiating myoblasts, and comparing the fraction of counts from single- to double-stranded sediments, relative to the mass of DNA within a single cell. In later studies (including this one), the extent of myogenic DNA damage is estimated using either population-scale measurements such as the comet assay; or using surrogate markers for DNA damage such as γH2AX or 53BP1. These markers work well for estimating and comparing the gross extent of DNA damage, but they do not allow exact enumeration of individual DNA strand breaks. Each γH2AX focus may not be indicative of an individual DNA strand break, and foci can also be found overlapping on micrographs, further confounding the analysis.

One possible method to enumerate DNA strand breaks could be a modification of the TUNEL assay. Free DNA ends would be labelled with biotin-conjugated nucleotides using the TdT enzyme. The chromatin would then be sheared into fragments either physically or enzymatically. The biotin-labelled fragments would be purified and labelled with universal primers. Quantitative PCR would then be used to enumerate the number of purified fragments, and thus the number of cleavage events. Such a methodology may even allow for mapping of the sites of DNA strand
breakage during terminal differentiation if it were complemented with DNA sequencing, and thus act as a direct test as to whether particular sites are cleaved during myogenesis, or whether genomic cleavage is truly random.

The origin of single stranded DNA breaks in myogenesis

During myogenesis, cells accumulate not only double-stranded DNA breaks, but also single-stranded breaks (Farzaneh, 1982; Coulton, 1992). Additionally, chemical inhibition of Poly ADP ribose polymerase (PARP), an enzyme which is crucial to single-stranded break repair, blocks myogenic differentiation (unpublished data from the Fearnhead lab). Together, this suggests that single-stranded breaks not only occur during myogenesis, but their resolution is necessary for the myogenic program.

This is problematic for the theory that CAD is the agent which is responsible for nuclear DNA cleavage during myogenesis, because CAD is capable only of producing double-stranded breaks (Hanus, 2008). If myogenesis is accompanied by single-stranded breaks, then the question must be asked; how are these lesions produced?

In order to investigate this, a method is needed to discriminate between double-stranded and single-stranded DNA breaks during myogenesis.

There are several ways this could be achieved. The Brdu assay, used in chapter 4, is routinely used as a measure of DNA synthesis. Normally, this assay is performed as follows: the synthetic nucleoside bromodeoxyuridine is supplied to cells in excess. Cells take up this nucleoside and
incorporate it into de novo DNA strands. At the end of the experiment, the cells are fixed for immunofluorescence imaging. A key step is that the fixed cells must be treated with a strong acid in order to unwind the nuclear DNA. This is necessary because the anti-Brdu antibody recognizes the internal nitrogenous base of Brdu, and this internal base structure must be exposed in order for the antibody to bind. However, if tracts of single-stranded DNA are present, the anti-Brdu antibody should be able to bind without the unwinding treatment. Thus the Brdu assay performed without the acidic treatment could be used as a direct readout for single-stranded DNA damage.

Interestingly, it has been shown that DNase gamma, another apoptotic endonuclease, is active during myogenic differentiation (Shiokawa, 2002). DNase gamma is capable of producing single-stranded DNA nicks (Mizuta, 2006). Whether the action of DNase gamma is actually required for myogenic differentiation has not yet been determined. A potent small molecule inhibitor of DNase gamma, DR396, has been developed (Yamada, 2011). This inhibitor may allow us to ask whether the activity of DNase gamma is responsible for inducing the single-stranded breaks seen during differentiation, and by extension whether DNase gamma is required for myogenic differentiation.

**DNA damage: a more direct induction**

In chapter 1, it was shown that irradiation of myoblasts produced dose-dependent DNA damage. This treatment also produced myogenic differentiation. In chapters 3 and 4, it was shown that inhibition of the checkpoint kinases wee1, chk1, and ATR also produced substantial DNA damage, accompanied by spontaneous myogenic differentiation. These results, as well as reports cited from the literature, suggest that DNA strand breaks are causal to spontaneous myogenic differentiation. However, it is always possible that each of these approaches produces myogenic
differentiation through some other, unrelated mechanism. Perhaps UV irradiation produces radical oxygen species, and these lead to mitochondrial permeabilization and conventional caspase-driven differentiation. Perhaps checkpoint kinase inhibition produces differentiation through some other effect, unrelated to DNA damage induction.

To address this criticism, a “cleaner” method of DNA damage will be used. There exist a wide range of endonucleases which can cleave genomic DNA into fragments of different average size, the size being dependent upon how frequent the cleavage motif is found within the chromatin. Ectopic expression of such endonucleases could be one strategy to test whether DNA damage itself, in isolation, is sufficient to drive differentiation. This methodology has the added advantage in that it allows a crude mapping of the sites of DNA damage, since the cleavage motif is known and can be compared to a full genome assembly. If it is shown that endonuclease-induced DNA damage can be used to directly induce differentiation, then the targeted sites can be compared to sites of known myogenic genes. If a correlation is found, then this indicates that particular sequences may be involved in triggering myogenic genes, and therefore that myogenic DNA damage is site-directed, despite the evidence presented in this thesis. If a random distribution of genomic cleavage is capable of inducing differentiation, then it argues for the theory that myogenic DNA damage is undirected, and that all that is needed is sufficient activation of DNA damage response complexes.

An alternative hypothesis: CAD-mediated gene amplification

A publication from 1985 (Wright) showed that forced expression of myogenic factors was able to directly induce myogenic differentiation. In essence, the activation of the myogenic program is determined by the expression of myogenic genes.
A recent report (Fischer, 2016) demonstrated that several genes; CDK4, NUP133, and MYO18B; undergo copy number amplification during myogenic differentiation in C2C12 myoblasts. Each of these gene products; CDK4, Nucleoporin 133, and Myosin-XVIIIb; are functionally involved in the process of myogenic differentiation (Hlaing, 2002; D’Angelo, 2012; Ajima, 2008). Interestingly, Fischer also showed that a portion of the amplified DNA is ‘expelled’ into the cytoplasm. This suggests that gene duplication, rather than transcriptional upregulation, is a strategy used to increase the levels of some genes during myogenic differentiation.

Taken together with the findings that both DNA strand breakage and the non-homologous end-joining enzyme DNA-PK are required for the differentiation program to proceed, another model of myogenic differentiation can be constructed:

During differentiation, certain myogenic gene sequences become amplified, leading to a higher copy number. These sequences remain in the nucleus, but are extrachromosomal. Strand breaks are produced by CAD or some other nuclease mechanism. The amplified sequences are then ligated into the genome by the non-homologous end-joining process. Unincorporated amplified sequences are expelled into the cytoplasm as a form of dosage compensation. To all conventional gene expression assays, this gene duplication would appear as though it were simply an increase in gene expression. Copy number amplification represents an alternative strategy to increase the expression of a given gene.

If this mechanism is true, then inhibition of DNA-PK, as performed in chapter 2, should prevent the accumulation of amplified sequences in the chromatin, as should inhibition of CAD. As a
consequence, a higher proportion of the amplified sequences should be found in the cytoplasm relative to the nucleus.

An interesting question is how this gene amplification takes place. Mechanisms of gene duplication can be difficult to study because these events usually happen sporadically, generally as a result of mistakes in DNA replication. Fischer (2016) speculate that the mechanism involved is due to multiple firing of replication origins and gene reduplication. This is where multiple copies of a gene are produced as a result of overlapping replication fork domains. It has been shown that such events are induced when CDK activity is dysregulated, particularly the activity of cyclin A/CDK1 and cyclin B/CDK1 complexes (Porter, 2008; Edgar, 2001).

In chapters 3 and 4, it was shown that inhibition of the checkpoint kinases wee1, chk1, and ATR each causes unscheduled replication origin firing and CDK-mediated DNA synthesis, and that this leads to spontaneous myogenic differentiation. In the literature it has been shown that wee1 and chk1 are both downregulated over the course of myogenic differentiation, and that the activity of ATR is inhibitory to myogenic differentiation (Sarkar, 2010; Fortini, 2012; Smith, 1998). Together, this suggests a mechanism in which downregulation of these checkpoint kinases during differentiation leads to unregulated CDK activity, multiple replication origin firing, and gene duplication. Together with the CAD-mediated strand breakage and DNA-PK-mediated ligation suggested earlier, this presents a mechanism through which myogenic gene duplication can occur (Diagram 1). If expression of myogenic genes is sufficient to drive differentiation, then this gene amplification mechanism could explain the requirement for strand breaks and DNA repair in myogenesis.
Diagram 1: A model of myogenic gene duplication and chromosomal incorporation. Through downregulation of CDK-inhibitory kinases wee1, chk1 and ATR, myogenic genes are reduplicated. These extrachromosomal myogenic genes are incorporated into the chromatin though CAD-mediated strand breakage, followed by DNA-PK-mediated ligation. Unincorporated genes are expelled to the cytoplasm as a form of dosage compensation.

This model of gene copy number amplification could explain the function of CAD-induced DNA strand breakage, as well as the transient increase in DNA-PK expression which is seen in fusion-competent myoblasts as compared to quiescent satellite cells and mature myotubes (Vahidi Ferdousi, 2014).

If CAD-induced gene amplification is a required event in the myogenic program, this would also settle the ‘cell autonomous’ verses ‘apoptotic signaling’ debate about the roles of caspases, since a cell autonomous caspase activity would be required to bring about these strand breaks. If this caspase-induced gene duplication model is correct, this may also provide a mechanism through which “killer” caspases can directly alter the genome.
Annexes
Annex I: Myogenic differentiation can be blocked by inhibition of voltage-dependent anion channel

Caspase-3 is required for myogenic differentiation. Since caspase-3 is transcribed as an inactive proenzyme, caspase-3 must be activated somehow during myogenesis. In apoptotic cell death, caspase-3 can be activated either by a death receptor driven caspase-8-dependent extrinsic pathway, or by a mitochondrial-driven caspase-9-driven intrinsic pathway. It has been shown that caspase-9 and Bcl-X\textsubscript{L} are required for myogenic differentiation (Murray, 2008). Because of this, it seems likely that the mitochondrial pathway of caspase activation is involved. However, direct evidence for such mitochondrial activation is scant and conflicting.

Bloemberg (2014) reported that in differentiating myoblast cultures, Bax and PUMA are upregulated in differentiating myoblast cultures concomitant with caspase activity, but that other pro-apoptotic mitochondrial indices were undetectable. Shaltouki (2007) reported PUMA upregulation and cytochrome C release in differentiating myoblasts. Bax has been found to be upregulated in regenerating muscle fibers, but this appears to be cancelled out by a concurrent upregulation of the anti-depolarization protein Bcl-2 (Tews & Goebel, 1997). Murray (2008) attempted to investigate mitochondrial membrane depolarization directly using TMRE staining combined with flow cytometry, but were unable to detect TMRE loss during differentiation.

Here, an inhibitor-based approach was used to investigate the role of mitochondrial depolarization in myogenesis. The Voltage-Dependent Anion Channel (VDAC) is key to mitochondrial
depolarization and cytochrome C release during apoptosis (Lemasters 2006, Tsujimoto 2002). The VDAC inhibitor DIDS (4,4'diisothicyanato-stilbene-2,29-disulfonic-acid) is used to block VDAC activity and prevent mitochondrial depolarization. DIDS prevents mitochondrial-induced apoptotic cell death in mouse myoblasts (Wang, 2015). Treatment with DIDS blocks myogenic differentiation in a dose-dependent manner (Figure 1), although at 50 and 100 µM this is due to cell toxicity, as an obvious decrease in cell density is apparent at these concentrations.

Figure 1: Effect of the VDAC inhibitor DIDS on myogenic differentiation. Myoblasts were treated with DIDS at the same time as serum withdrawal. The cells were allowed to fuse for 72 hours, before being fixed and stained for myosin heavy chain. A: Representative micrographs of the effect of DIDS on myogenic fusion. B: Fusion index calculated for each concentration of DIDS. Data represent means ± SEM of three independent experiments.
Annex II: Genotoxic agents block myogenic differentiation, but are toxic

Puri (2002) postulate a differentiation checkpoint activated by genotoxic stress. This is in contradiction with my claim that DNA damage itself induces myogenic differentiation. However, the chemical agents which they used to induce DNA damage in that study (etoposide, cisplatin, and MMS) all have substantial known effects on both gene transcription and cell cycle progression, each of which can account for the block in fusion seen without the need to invoke a ‘differentiation checkpoint’.

To confirm the observations of Puri, I tested the effect on myogenic differentiation of the topoisomerase poisons etoposide and doxorubicin (Figure 1). Both drugs exhibit dose-dependent inhibition of myogenic differentiation, although it is evident that this is due to cellular toxicity in both cases, with many nuclei showing abnormal necrotic and karyohectic features.
Figure 1: Genotoxic agents block myogenic differentiation, but this is due to cellular toxicity.

Myoblasts were treated with a concentration range of doxorubicin or etoposide, differentiated for 72 hours, then fixed and stained for myosin heavy chain. A: Doxorubicin. B: Etoposide.
Annex III: Tocris kinase inhibitor library screen

Below is the full list of fusion indices calculated from the Tocris screen. Hit compounds are highlighted. Hits were defined as compounds which produced a statistically-significant difference in fusion index relative to DMSO control. Statistical significance was assessed using ANOVA followed by Dunnett’s post-hoc test.

Table 1: Effect of Tocris compounds on myogenic fusion.

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Annex IV: Clinical inhibitors of EGFR block myogenic differentiation

One of the hit compounds from the Tocris library was GW-583340. This represents an interesting hit because it is a very close structural analog of the chemotherapeutic compound Lapatinib (Diagram 1):

Diagram 1: Comparison of the structures of GW-583340 and Lapatinib.

Lapatinib is an EGFR tyrosine kinase inhibitor which is very commonly used to target solid tumors. That GW-583340 has such a profound effect on myogenesis suggests that the related drug Lapatinib may have previously uncovered side-effects in patients, perhaps related to chemotherapy-associated weight loss.

In this annex, I tested the hypothesis of whether Lapatinib and perhaps other EGFR-targeting therapeutics could block myogenic differentiation in vitro; and if so, whether such an inhibition could occur at clinically relevant concentrations.
Here I present evidence that several EGFR inhibitors including the clinical chemotherapeutic compound lapatinib and gefitinib exhibit a profound inhibition of skeletal muscle differentiation in vitro. This inhibition is independent of generalized cell toxicity, but is associated with a large reduction in myogenic fusion. The evidence presented here suggests the possibility that EGFR inhibition therapy may actually enhance the progression of cachexia during cancer treatment through the degradation of normal muscle regeneration processes.

**Figure 1 (overleaf): EGFR inhibitors block myogenic differentiation.** Cells were treated with each of the EGFR targeting compounds and induced to differentiate for 72 hours. A: Example immunofluorescence micrographs of myoblasts treated with each compound. B: Fusion dose response to each of the compounds tested. C: Effect of each compound on cell density. Gefitinib, Lapatinib, and GW-583340 have fusion-inhibitory activity at concentrations that do not produce a decrease in cell density. Data represent means ± SEM of three independent experiments.
The data here suggest that two clinical EGFR tyrosine kinase inhibitors; Gefitinib and Lapatinib, both inhibit myogenic differentiation *in vitro*. Below is a table of the calculated IC$_{50}$ and % inhibition values calculated for each drug in this study, compared to the reported IC$_{50}$ against either EGFR/Her1 or Her2/ErbB2. Each of the compounds which showed activity against myogenic fusion have activity against EGFR/Her1. Gefitinib has activity against myogenic fusion, and is a weak inhibitor of Her2/ErbB2. This suggests that it is primarily EGFR/Her1 activity which is required for myogenic differentiation. BIBX-1382 has little activity against myogenic fusion, despite being a potent inhibitor of EGFR/Her1. This result may be due to the fact that BIBX is structurally unrelated to the hit compounds and may have solubility or cell permeability issues in this system.

Table 1: IC$_{50}$ and % inhibition values calculated for each compound in this study, compared to the IC$_{50}$ values reported in the literature

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC$_{50}$ against myoblast differentiation (nM)</th>
<th>% inhibition of myoblast differentiation (nM)</th>
<th>IC$_{50}$ against EGFR/Her1 (nM)</th>
<th>IC$_{50}$ against Her2/ErbB2 (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AG-490</td>
<td>10 nM</td>
<td>21.98</td>
<td>2,000 nM (cell free) (Gazit)</td>
<td>13,500 nM (cell free) (Gazit)</td>
</tr>
<tr>
<td>BIBX-1382</td>
<td>&gt;3,000 nM</td>
<td>26.24</td>
<td>3 nM (cell free) (Solca)</td>
<td>3,400 nM (cell free) (Solca)</td>
</tr>
<tr>
<td>Gefitinib</td>
<td>200 nM</td>
<td>63.99</td>
<td>33 nM (in cellulo) (Wakeling)</td>
<td>3,700 (in cellulo) (Wakeling)</td>
</tr>
<tr>
<td>GW-583340</td>
<td>60 nM</td>
<td>73.55</td>
<td>10 nM (in cellulo) (Gaul)</td>
<td>14 nM (in cellulo) (Gaul)</td>
</tr>
<tr>
<td>Lapatinib</td>
<td>15 nM</td>
<td>78.7</td>
<td>10.8 nM (cell free) (Rusnak)</td>
<td>9.2 nM (cell free) (Rusnak)</td>
</tr>
</tbody>
</table>
Pharmacokinetic Relevance

Hudachek (2013) reported that a scaled clinical dose of Lapatinib at 30 mg/kg in mice resulted in its accumulation in skeletal muscle tissue at a concentration of 2 µM in a scaled mouse model. The effect of Lapatinib on C2C12 myogenisis was observed at concentrations as low as 10 nM, with an IC₅₀ of approximately 15 nM. Since this is much lower than the reported concentration at which Lapatinib accumulates in skeletal muscle, the findings presented in this thesis are at pharmacokinetically-relevant concentrations.
A timeline of important publications in the field

1982 - Farzaneh: Differentiating myoblasts accumulate DNA strand breaks.


1985 - Farzaneh: Myogenic DNA damage is not produced through a failure of DNA repair, but through active strand breakage.


1988 - Dawson & Lough: DNA damage is repaired over the course of differentiation.


1992 - Coulton: DNA damage occurs within regenerating muscle fibers in live mice.

1994 - Jost & Jost: **Base-excision DNA repair is active in differentiating myoblasts.**


1995 - Sandri: **DNA strand breaks are detected in muscle sections after exercise**


2002 - Shiokawa: **A nuclease activity is responsible for myogenic DNA damage.**


2002 - Fernando: **Caspase-3 is required for myogenic differentiation.**


2002 – Puri: **DNA damage initiates a myogenic checkpoint which halts differentiation until the damage is resolved.**

2003 - Hossain: **Double-stranded break-inducing agents induce differentiation in drosophila myoblasts.**


2008 - Murray: **Caspase-9 and Bcl-XL are required for myogenic differentiation.**


2009 - Ikeda: **Caspase-3 and caspase-9 are detected in developing embryonic skeletal muscle.**


2009 - Sudo: **Regenerating muscle fibers contain TUNEL-positive nuclei centrally & in the sarcolemma, indicating DNA damage in regenerative cells.**


2010 - Larsen: **CAD is required for myogenic differentiation.**

2013 - Hochreiter-Hufford: **Apoptotic myoblasts stimulate differentiation through phosphatidyserine-contact signaling**


2014 - Bloemberg: **Bax and PUMA are upregulated concomitant with caspase activity during differentiation.**


2016: Al-Khalaf: **XRCC1-mediated DNA repair is require for myoblast differentiation**

References


Connolly PF, Jäger R, Fearnhead HO. (2014) New roles for old enzymes: killer caspases as the engine of cell behavior changes. 5:149.


Klimpel, GR (1996) Immune Defenses. Chapter 50, Medical Microbiology, 4th edition. University of Texas Medical Branch at Galveston; Galveston TX.


List of publications, conference talks and posters generated from the work in this thesis

Publication: Connolly PF, Fearnhead HO (2016) DNA-PK activity is associated with caspase-dependent myogenic differentiation. FEBS J. [Epub ahead of print].


Publications
New roles for old enzymes: killer caspases as the engine of cell behavior changes

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INTRODUCTION

Caspases are intracellular cysteine proteases that cut after specific aspartic acid residues. In mammals, 18 caspases have been characterized (Table 1), the majority of them playing roles in mediating apoptotic cell-decomposition (Cohen, 1997). During apoptosis, these “killer caspases” cleave numerous cellular proteins, and they are the primary effectors responsible for taking apart the cell during apoptosis, playing a major proteolytic role in the disassembly of the nucleus and the cytoskeletal structure (Lüthi and Martin, 2007).

Caspases are present in all cells as inactive zymogens, called procaspases. They are activated through cleavage to generate the subunits that form an active caspase (Pop and Salvesen, 2009). At the apex of the activation cascade are the so-called initiator caspases (Table 1). Upon exposure to an apoptotic stimulus they become recruited to specific adaptor proteins which then assemble into activation platforms, which are large multimeric protein complexes mediating activation of the initiator caspases (reviewed in Mace and Riedl, 2010). Initiators activate downstream effectors which rapidly disassemble the cell. The ability of these caspases to kill cells is controlled, in part, by the inhibitor of apoptosis proteins (IAPs) which bind to active caspases and either inhibit proteolytic activity or induce ubiquitin-mediated caspase degradation (Mace et al., 2010).

Different activation platforms classify the main apoptotic pathways. The extrinsic pathway is initiated at the cell membrane by ligands of receptors of the tumor necrosis factor (TNF) receptor family. Ligand-binding leads to assembly of the death-inducing signaling complex (DISC) containing these receptors. Caspase-8 and -10 are recruited to the DISC and then activated, in a process that requires the adaptor protein FADD. The intrinsic pathway is initiated by mitochondria, whose outer membranes become permeable to cytochrome c upon certain cellular stresses. Released cytochrome c then binds to the adaptor protein APAF-1 which subsequently assembles into a large heptameric protein complex, the so-called apoptosome, which is the activation platform for caspase-9. The release of cytochrome c is controlled by proteins of the Bcl-2 family (Tait and Green, 2010). Concomitant with release of cytochrome c, other small proteins may be released, some of which block IAPs allowing for unrestrained caspase activation.

The majority of studies of caspases have focused on their roles as cell killers. There were some notable exceptions describing caspase-dependent cellular differentiation processes that involve denucleation or other degenerative events (Fernando and Megeney, 2007), but these appear to represent a limited or frustrated apoptosis, rather than a fundamentally different process. More recently, the study of apoptotic caspases has broadened to include caspase-dependent paracrine signaling from apoptotic cells to explain how apoptotic cells alter the behavior of surrounding cells (Li et al., 2010a).

However, it has also emerged that apoptosis-associated caspases play non-apoptotic roles and that they are not simply destructive. Examples of these are cell differentiation (Fernando and Kelly, 2002), embryonic development (Miura, 2012; Suzanne and Steller, 2013), motility (Barbero et al., 2009), and compensatory proliferation (Fan and Bergmann, 2008). Within these processes, the “killer” caspases clearly do not cause cell demoli-tion. This gives caspases an entirely new role in determining the fate or behavior of cells. Thus, caspases drive in a far wider range of cellular behaviors than previously known.

There are several theories to explain how apoptotic caspases can lead to non-apoptotic outcomes (Figure 1). In one mechanism, the “cell-autonomous” or “direct” model, caspase activity leads to altered cell behavior through the modulation of regulatory networks, such as through cleavage of cell cycle repressors to alter cell proliferation (Schwerk and Schulze-Osthoff, 2003; Woo et al., 2003), activation of gene transcription to induce skeletal cell behavior changes...
Table 1 | Roles of caspases.

<table>
<thead>
<tr>
<th>Caspase</th>
<th>Conventional role</th>
<th>Other roles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caspase-1</td>
<td>Inflammatory response (Kuranaga and Miura, 2007)</td>
<td>Unknown</td>
</tr>
<tr>
<td>Caspase-2</td>
<td>Apoptosis (Initiator) (Inoue et al., 2009)</td>
<td>DNA damage response (Fava et al., 2012)</td>
</tr>
<tr>
<td>Caspase-3</td>
<td>Apoptosis (Executioner) (Kuranaga and Miura, 2007)</td>
<td>Differentiation of many cell types (see Table 2)</td>
</tr>
<tr>
<td>Caspase-5</td>
<td>Inflammatory response (Fuchs and Steller, 2011)</td>
<td>Possibly tumor suppression (Soung et al., 2008)</td>
</tr>
<tr>
<td>Caspase-6</td>
<td>Apoptosis (Initiator) (Inoue et al., 2009)</td>
<td>Unknown</td>
</tr>
<tr>
<td>Caspase-7</td>
<td>Apoptosis (Executioner) (Fuchs and Steller, 2011)</td>
<td>Unknown</td>
</tr>
<tr>
<td>Caspase-8</td>
<td>Apoptosis (Executioner) (Fuchs and Steller, 2011)</td>
<td>Embryonic development (Suzanne and Steller, 2013), motility (Helfer et al., 2006), tumor metastasis (Barbero et al., 2009), T-cell proliferation (Kennedy et al., 1999), cell cycle regulation (Zhang et al., 2001; Hashimoto et al., 2011), bacterial infection response (Kayagaki et al., 2011)</td>
</tr>
<tr>
<td>Caspase-9</td>
<td>Apoptosis (Initiator) (Cohen, 1997)</td>
<td>Differentiation of many cell types (see Table 2)</td>
</tr>
<tr>
<td>Caspase-10</td>
<td>Apoptosis (Initiator) (Inoue et al., 2009)</td>
<td>Immune response to dsRNA (Takahashi et al., 2006), possible tumor suppressor (Park et al., 2002)</td>
</tr>
<tr>
<td>Caspase-11</td>
<td>Inflammatory response (Li et al., 2007)</td>
<td>Cell migration (Li et al., 2007)</td>
</tr>
<tr>
<td>Caspase-12</td>
<td>Inflammatory response (Leulier et al., 2000)</td>
<td>Unknown</td>
</tr>
<tr>
<td>Caspase-14</td>
<td>Keratinocyte differentiation (Zermati et al., 2001)</td>
<td>Unknown</td>
</tr>
<tr>
<td>Caspase-15</td>
<td>Apoptosis (Initiator) (Eckhart et al., 2005)</td>
<td>Unknown</td>
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<tr>
<td>Caspase-16</td>
<td>Unknown, phylogenetic association with caspase-14</td>
<td>Unknown</td>
</tr>
<tr>
<td>Caspase-17</td>
<td>Unknown, phylogenetic association with caspase-3</td>
<td>Unknown</td>
</tr>
<tr>
<td>Caspase-18</td>
<td>Unknown, phylogenetic association with caspase-8</td>
<td>Unknown</td>
</tr>
</tbody>
</table>

The majority of caspases are primarily involved in programmed cell death, and the minority are primarily involved in the generation of immune responses. Most have been discovered to play other roles as well.

In this cell autonomous model, it is as yet unclear how apoptotic caspase activity is harnessed for non-apoptotic purposes without killing the cell, although work in Drosophila melanogaster has begun to unravel this problem. For example, a recent report of caspase activation in Drosophila proposes a model in which both the magnitude and rate of caspase activation is controlled, which can give rise to high (apoptotic) levels of caspase activity as well as low (non-apoptotic) levels of activity (Florentin and Arama, 2012). It is also possible that, unlike the traditional model where executioner caspases are only activated upon receipt of a cell stress signal, there is a constant basal level of activated caspases within the cell, but these are normally held in check by inhibitory mechanisms. Such basal levels of caspase activity have been found in the context of cell behavior changes in glioblastoma cells, where low levels of constitutively-active caspase-8 and -3 are found to be necessary for cell migration and invasion (Gdynia et al., 2007). Along with this, relatively high levels of caspases activity may be tolerated if they can be sequestered within their target organelle or sub-cellular region, as is observed in the dendritic pruning of neurons (Williams et al., 2006), in spermatid individualization in Drosophila (Arana et al., 2007; Kaplan et al., 2010), and in the nuclear degradation of keratinocytes (Weil et al., 1999).

In the “non-autonomous” or “indirect” model to explain the role of caspases in non-apoptotic processes, the caspase activity is localized within apoptotic cells, catalyzing the generation of secretory paracrine signaling factors or enabling cell surface-mediated signaling (Hochreiter-Hufford et al., 2013). This model is indirect...
in that the caspase activity is associated with one cell, while the downstream effect is induced in another cell by an intercellular signaling event. In this model the caspase-mediated non-apoptotic effects do not necessarily require the survival of the “caspase-active” cell, as apoptotic cells are still quite capable of signaling to their environment (Jäger and Fearnhead, 2012).

Here, we review the major non-apoptotic roles of caspases discovered to date, and discuss these findings in light of the direct and indirect theories of caspase signaling, with a particular focus on skeletal muscle. This is a rapidly advancing field of study, and a summation of the current state of the field is necessary.

TISSUE REPAIR AND REGENERATION
Caspases are key players in the homeostatic balance between apoptosis and regeneration used to maintain tissue structure and function. In response to injury, dead cells engage in a signaling behavior which drives the proliferation of cells at the periphery of the site of injury until damaged portion of tissue is replaced with a new section of the same size and shape (Bergmann et al., 2010). The role of caspases in repair and regeneration has been demonstrated in several different experimental models.

In the simple metazoan Hydra, surgical-induced injury produces an apoptotic response which stimulates a compensatory proliferative mechanism in surrounding progenitor cells. Treatment with pan-caspase inhibitors abolishes this regenerative response (Cikala et al., 1999; Chera et al., 2009). Regeneration in the amphibian Xenopus requires caspase-mediated events (Tseng et al., 2007), as does tissue regeneration in planaria (Fuchs and Steller, 2011), and the regeneration of newt forelimbs (Vlaskalin et al., 2004).

Regeneration of mammalian tissue is never so dramatic but some tissues, like the liver, can undergo remarkable regeneration after injury (Taub, 2004). Liver regeneration and the healing of skin wounds is impaired in caspase-3 and -7 deficient mice, showing that the role of caspases in regenerative processes is conserved in mammals (Li et al., 2010a).

Paracrine molecules secreted by the apoptotic cells appear to be important in caspase-dependent regeneration. Prostaglandin E2 is one such a molecule, as its production pathway is directly controlled by caspase-3 (Boland et al., 2013), and has a wide number of roles in regeneration and proliferation (Castellone et al., 2005; Goessling et al., 2009; Morata et al., 2011; Beaulieu et al., 2012; Boland et al., 2013). This effect is exerted through transient activation of the Wnt-β-catenin pathway via binding to members of the EP receptor family (Goessling et al., 2009).

Lysophosphatidylcholine (LPC) is another molecule which mediates caspase-activity-induced regenerative responses. It is produced by apoptotic cells, and its presence in the interstitial medium acts as an attraction signal to phagocytes (Lauber et al., 2003). Moreover, LPC induces the differentiation of keratinocytes, which is a necessary step in the wound healing response in skin (Ryborg et al., 2004).

Sphingosine-1-phosphate is another molecule that is secreted by apoptotic cells and is a chemoattractant signal for immune cells (Brecht et al., 2011). It is produced by the enzyme ceramide, and is a signal that drives growth arrest and differentiation, as well as cell migration and adhesion (Mao and Obeid, 2008), all of which are involved in wound-healing responses.

Fractalkine (CX3CL1) is a large peptide that engages in pro-survival functions in many cell types (White and Greaves, 2012) and is released from apoptotic cells in a caspase-dependant process (Truman et al., 2008). Fractalkine is normally associated with immune cell chemotaxis (Chazaud et al., 2003), but it is also known that soluble fractalkine promotes both migration of endothelial cells and differentiation of osteoblasts (Koizumi et al., 2009). This makes it another potential paracrine signaling factor released by apoptotic cells to modulate tissue regeneration.

From the examples described, it is seen that caspase activity in a dying cell can indirectly induce compensatory proliferation of neighboring cells as part of the regenerative response to injury. Thus, regenerative processes conform to the indirect, apoptotic cell-driven model of caspase function in non-apoptotic processes. However, caspases can also regulate cell proliferation in a cell-autonomous manner through the cleavage of cell cycle regulators.

LYMPOCYTE PROLIFERATION
There are hundreds of confirmed caspase substrates (Lüthi and Martin, 2007; Johnson and Kornbluth, 2008), although the functional significance of cleavage is often uncertain. Among this large group there are several key cell cycle regulators (Hashimoto et al., 2011) and a series of studies have implicated both initiator and effector caspases in the control of the cell cycle of lymphocytes. Because of this, abnormal caspase activity can lead to either depressed or hyperactive cell proliferation.

Through the DISC adaptor protein FADD, caspases-8 and -10 play a role in cell proliferation (Imtiyaz et al., 2009). Peripheral T-cells from FADD deficient mice show a profound impairment of proliferation once they are activated by mitogens or antigens, leading to a reduced number of mature T-cells (Zhang et al., 1998). This inhibition of proliferation results from a failure to enter the cell cycle at the beginning of S-phase due to abnormal expression of cyclin-dependent kinases (Zhang et al., 2001).
Pharmacological inhibition of caspases prevents T-cell proliferation in vitro supporting the idea that caspase activity is required for proliferation (Kennedy et al., 1999). The cell cycle role for caspases may not be limited to T-cells as impaired T-cell, B-cell, and NK-cell proliferation is seen in immune-deficient humans with caspase-8 defects (Chun et al., 2002).

As well as caspases-8 and -10, caspase-3 has also been found to play a role in regulating cell proliferation. Proliferative brain cells were found to contain active caspase-3, localized in the nucleus (Oommen et al., 2005). In lymphoid cells, caspase-3 supports the proliferation through cleavage of the CDK inhibitor p27 (Frost et al., 2001). In these examples, caspase-mediated stimulation of proliferation appears to be a cell-autonomous event. On the other hand, proliferating cells utilize caspases in a non-apoptotic capacity to downregulate cell cycle inhibitors which normally keep the cell in a quiescent state (Zhang et al., 2001; Woo et al., 2003; Lamkanfi et al., 2007). For example, caspase-3 can exert a strongly anti-proliferative effect in B-cells through cleavage of p21 and caspase-3 knockout mice show a hyperproliferative phenotype in their B-cells (Woo et al., 2003). Thus, killer caspases seem to be able to exert both positive and negative regulation of cell proliferation through selective cleavage of cell cycle regulators without necessarily inducing apoptosis.

Caspase-1, which is involved in toxin-sensing (Li et al., 1995; Franchi et al., 2009), and caspase-11, involved in the production of inflammatory factors (Kayagaki et al., 2011) have long been associated with inflammation and immunity and are not central to cell death processes. However, it has more recently been discovered that apoptotic caspases also have roles in immunity. This can be through their role in the differentiation programs of immune cells, such as with caspase-8 paralog Dredd (Leulier et al., 2000), or through the modulation of the immune response itself, such as with caspase-12 (Saleh et al., 2004) and caspase-3 paralog ced-3 (Aballay and Ausubel, 2001). Such modulation may occur through the generation of inflammatory and anti-inflammatory factors (Kuranaga and Miura, 2007) or through their role in the apoptosis of infected cells.

**DIFFERENTIATION**

Caspases engage in irreversible signal transduction (Kuranaga, 2012). Such irreversible signaling mechanisms are suitable for guiding cell fate choices, such as differentiation. Indeed, such caspase signaling has been found to play important roles in the terminal differentiation programs of several cell types, both in early development, and in tissue regeneration.

The first cell types in which caspases were found to have a direct role in differentiation had one feature in common: their differentiation programs bore a strong resemblance to apoptosis. For example, during terminal differentiation of the lens fiber cells degenerative processes including organelle degradation, chromatin condensation, and DNA fragmentation all occur, and are mediated by the activity of caspases (Ishizaki et al., 1998). The time required for this apoptosis-like process is much longer than that required for caspase-driven cell death, suggesting a more controlled and meticulous version of the same general procedure. Soon after this, erythrocytes and keratinocytes were also found to utilize caspase activity in their terminal differentiation programs (Eckhart et al., 2000; Zermati et al., 2001).

Subsequently, it was found that caspases also play roles in differentiation programs that bore no major similarity to apoptosis. An example of this is the differentiation of peripheral blood monocytes into macrophages, which requires the activation of the caspases-3, -8, and -9 for differentiation (Sordet et al., 2002). Deletion of caspase-3 limits the cytokine-induced differentiation of hematopoietic stem cells (Janzen et al., 2008) and the differentiation ability of iPSCs is enhanced by transient induction of caspase activity (Li et al., 2010b). Several other caspase-dependent cell differentiation programs have been discovered, including those of skeletal myoblasts, osteoblasts, spermatids, placental trophoblast, and embryonic and neural stem cells (Table 2).

A well-studied example of caspase-stimulated in vitro differentiation is that of mouse muscle myoblasts into multinucleated myotubes (Fernando and Kelly, 2002; Murray et al., 2008; Larsen et al., 2010). In this model caspase-3 mediates activation in differentiating mouse myoblasts of a specific DNase called CAD (Larsen et al., 2010). Normally CAD (DFF40 in humans) is bound to a chaperone called ICAD (DFF45) that inhibits the nuclease activity of CAD. Caspase-3 mediated cleavage of ICAD releases CAD, allowing it to cleave DNA. This is a key step in the generation of oligonucleosomal DNA fragments seen in apoptosis. Perhaps surprisingly, activation of CAD occurs in differentiating myoblasts and RNAi directed against CAD causes profound inhibition of myoblast differentiation. Larsen et al. propose that CAD-dependent activation of p21 expression is the key event explaining this defect as p21 expression is an early and necessary event in myoblast differentiation (Larsen et al., 2010). In other words, it is proposed that caspase-3 drives non-apoptotic outcomes by inducing expression of specific genes. In addition, Fernando et al. showed that microinjection of active caspase-3 induced expression of muscle specific genes (Fernando and Kelly, 2002). These two reports support the idea that caspase activity is present in the differentiating myoblast (the direct/autonomous model). Caspase-3 activation during differentiation requires caspase-9 and is blocked by overexpression of Bcl-XL (Murray et al., 2008), which implicates the intrinsic or mitochondrial apoptotic pathway, although the role of cytochrome c release or Apaf-1 in this differentiation has not been conclusively demonstrated.

Although this seems like strong evidence for cell-autonomous model of caspase action, there are also data to support non-autonomous roles for caspase activity in muscle differentiation. It has been found that myoblast fusion is driven by apoptotic cells through a phosphatidyserine-mediated activation of the BAI1 receptor. During apoptosis, caspase-dependent presentation of phosphatidyserine (PS) on the surface of dying cells is an important “eat me” signal for phagocytes and so plays a central role in the clearance of apoptotic bodies. Adding apoptotic cells to cultures where caspase activity has been abolished with pharmacological inhibitors restores myoblast fusion, and adding annexin V, a PS-binding protein, blocks myoblast fusion (Hochreiter-Hufford et al., 2013). This finding thus supports the non-cell-autonomous model, in that fusion is driven by caspase-mediated presentation of cell-surface signaling factors on apoptotic cells. It may be that both cell autonomous and...
Table 2 | Caspase involvement in the differentiation programs of several cell types.

<table>
<thead>
<tr>
<th>Differentiation morphology</th>
<th>Cell type</th>
<th>Caspase(s)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apoptosis-like</td>
<td>Erythrocyte</td>
<td>Caspase-2, -3, -9</td>
<td>Zermati et al., 2001</td>
</tr>
<tr>
<td></td>
<td>Keratinocyte</td>
<td>Caspase-3, -14</td>
<td>Weil et al., 1999; Eckhart et al., 2000</td>
</tr>
<tr>
<td></td>
<td>Epithelial lens</td>
<td>Caspase-3</td>
<td>Ishizaki et al., 1998</td>
</tr>
<tr>
<td></td>
<td>Megakaryocyte</td>
<td>Caspase-3, -9</td>
<td>De Botton et al., 2002</td>
</tr>
<tr>
<td>Non-apoptosis-like</td>
<td>Macrophage</td>
<td>Caspase-3, -8, -9</td>
<td>Sordet et al., 2002; Kang and Ben-Moshe, 2004</td>
</tr>
<tr>
<td></td>
<td>Skeletal myoblast</td>
<td>Caspase-3, -9</td>
<td>Fernando and Kelly, 2002; Murray et al., 2008; Larsen et al., 2010</td>
</tr>
<tr>
<td></td>
<td>Neuron</td>
<td>Caspase-3, -1</td>
<td>Fernando et al., 2005; Vaisid et al., 2005</td>
</tr>
<tr>
<td></td>
<td>Glial cell</td>
<td>Caspase-3</td>
<td>Oomman et al., 2006</td>
</tr>
<tr>
<td></td>
<td>Osteoblast</td>
<td>Caspase-2, -3, -8</td>
<td>Mogi and Togari, 2003</td>
</tr>
<tr>
<td></td>
<td>Placental trophoblast</td>
<td>Caspase-8</td>
<td>Black et al., 2004</td>
</tr>
<tr>
<td></td>
<td>Embryonic stem cell</td>
<td>Caspase-3</td>
<td>Fujita et al., 2008</td>
</tr>
<tr>
<td></td>
<td>Hematopoietic stem cell</td>
<td>Caspase-3</td>
<td>Janzen et al., 2008</td>
</tr>
<tr>
<td></td>
<td>Spermatid</td>
<td>Drice (Caspase-3), Dredd (Caspase-8), Dronc (Caspase-9)</td>
<td>Arama et al., 2003, 2007; Huh et al., 2004; Kaplan et al., 2010</td>
</tr>
<tr>
<td></td>
<td>Odontoblast</td>
<td>Caspase-7</td>
<td>Matalova et al., 2013</td>
</tr>
</tbody>
</table>

Some programs follow a “frustrated apoptosis” phenotype, while others have a distinctly non-apoptotic-like morphology.

non-autonomous roles for caspases are important in myoblast differentiation.

A question that arises with the cell-autonomous model of caspase activity; how is this activity prevented from progressing to apoptosis? The convention is that activation of apoptotic caspases is an irreversible threshold event, leading to a runaway process of proteolytic cleavage, culminating in apoptotic cell death. If caspases truly are activated within differentiating cells themselves, there must be some mechanism for restraining, sequestering, or otherwise preventing this activity from killing the cell. Members of the IAP family are important caspase regulators (Mace et al., 2010), but there is so far little evidence that any of these proteins regulates caspase activity during muscle differentiation. Kaplan et al. showed that in spermatids there is a gradient of the giant IAP protein, dBRUCE, that establishes a gradient of caspase activity during the process of spermatid individualization in Drosophila (Kaplan et al., 2010). There is also other evidence for caspase localization being important during differentiation (Table 3). Interestingly, myogenin expressing satellite cells from young donors display active caspase-3 only at the nucleus, whereas myogenin expressing satellite cells from aged donors contain active caspase-3 both at the nucleus and at the cytoplasm. The satellite cells from aged donors also show a higher level of apoptotic cell death and together these data suggest a model in which the failure to properly localize active caspase-3 leads to satellite cell death and impaired muscle regeneration as we age (Fulle et al., 2013).

Moving to in vivo models of muscle differentiation, the role of caspases in regeneration becomes less clear. In caspase-9 (Hakem et al., 1998; Kuida et al., 1998) and caspase-3 (Kuida et al., 1996) knock-out mice embryonic myogenesis appears normal so the role of caspase-9 and caspase-3 in muscle differentiation in vivo at first appears unlikely. However, besides prenatal myogenesis, there is a distinct postnatal muscle development process as well as repair and regeneration processes in adult muscle that have not been evaluated in the caspase knock-out mice. Defects in these processes underlie a range of muscular dystrophies and age-related sarcopenia. In some instances, defects that have profound effects on muscle regeneration do not affect embryonic muscle development. For example, mice lacking caveolin-3 or expressing a Pro104Leu mutation in caveolin-3 (a model for human Limb Girdle Muscular Dystrophy 1C) show normal muscle development but muscle degeneration after 8 weeks of age (Hagiwara et al., 2000; Sunada et al., 2001). It is therefore possible that caspase-driven processes are important primarily in regeneration of adult muscle rather than muscle development but defects have not been observed in caspase deficient mice because of the perinatal lethality associated with these knock outs.

Table 3 | Compartmentalization of caspases has been found to be used in the differentiation programs of several cell types.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Caspase</th>
<th>Form of compartmentalization</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platelet</td>
<td>Caspase-3</td>
<td>During platelet formation, caspase activity is localized in punctate bodies within the cytoplasm (De Botton et al., 2002)</td>
</tr>
<tr>
<td>Lens cell</td>
<td>Caspase-3</td>
<td>Partial localization to equatorial epithelium (Weber and Menko, 2005)</td>
</tr>
<tr>
<td>Spermatid</td>
<td>Drice (Caspase-3), Dredd (Caspase-8), Dronc (Caspase-9)</td>
<td>Localized within the cystic bulge of the cytoplasm (Kaplan et al., 2010)</td>
</tr>
<tr>
<td>Keratinocyte</td>
<td>Caspase-3</td>
<td>Probable localization in the nucleus during enucleation (Okuyama et al., 2004)</td>
</tr>
<tr>
<td>Glia</td>
<td>Caspase-3</td>
<td>Active caspase localized to the nucleus (Oomman et al., 2005, 2006)</td>
</tr>
</tbody>
</table>
Activation of caspase-8 by TNF induces apoptosis and blocks muscle regeneration in in vivo models of cachexia (Moresi et al., 2008, 2009), data that also appears inconsistent with a model in which caspase activity is required for differentiation. The conflicting reports of the role of caspases in muscle differentiation may be reconciled by a model in which caspase-8 induces high levels of effector caspase activity that kill cells while differentiation is associated with lower effector caspase activity. Just such a switch between death and differentiation has been reported in Drosophila models (Florentin and Arama, 2012). Alternatively, TNF-dependent caspase activation may result in a different localization of active caspases compared to caspase activation associated with differentiation as discussed above for young and aged satellite cells (Fulle et al., 2013).

It is also possible that a particular cellular differentiation process involves more than one caspase-driven step. Muscle differentiation may represent an example of this, with caspase signaling from apoptotic cells as well as caspase activity in the differentiating cells. Differentiating myoblasts may even rely on their caspase activity to drive more than one process during differentiation. Larsen et al. present compelling evidence that caspase-3 mediated DNA damage drives changes in gene expression that are required for myoblast differentiation (Larsen et al., 2010). Others have argued that primary consequence of preventing caspase activation in differentiation is a failure of myoblast fusion (Murray et al., 2008). It is possible that caspases contribute to myoblast fusion by influencing cell motility, as this is required for both muscle development (Brand-Saberi et al., 1996; Molkentin and Olson, 1996) and regeneration (Seale and Rudnicki, 2000).

MOTILITY AND METASTASIS

Cellular locomotion essentially involves the continuous deformation and manipulation of the cytoskeleton to achieve movement. Caspases are the major manipulators of cytoskeletal structure during apoptosis, so it is conceivable that caspases could also have a role in enabling cell motility. In support of this model, in vitro studies have shown that caspase-8-knockout mouse embryonic fibroblasts are both motility-defective, and unable to form proper lamellipodia (Helfer et al., 2006). It is thought that caspase-8 engages in a multiprotein complex with calpain to cleave focal adhesion substrates (Helfer et al., 2006).

Additionally, the embryonic lethality of caspase-8 homozygous knockout mice has been attributed to the failure to develop a functional circulatory system through a defect in endothelial cell migration (Kang and Ben-Moshe, 2004). It is as yet unknown how caspase-8 mediates migration. It could be mediated through activation of downstream effector caspases like caspase-3, leading to modification of the cytoskeleton, or it could act through a separate pathway that does not involve executioner caspases. There is even evidence suggesting that the catalytic activity of caspase-8 is not required for its effects on cell motility (Senft et al., 2007). In addition to caspase-8, caspase-3 has also been implicated in cell motility. Pharmacological inhibition of caspase-3 activity reduces cancer cell motility and invasiveness (Gdynia et al., 2007).

Metastasis of tumor cells involves cellular migration and invasion of tissues, and the subsequent growth of secondary tumors at distant sites. Normally, cells are unable to escape into systemic circulation, as detachment from their basement matrix induces cell death through anoikis or amorphosis (Mehlen and Puisieux, 2006). However, when apoptosis is compromised through silencing of the downstream effector caspase-3, caspase-8 can act to promote metastasis. In this state, caspase-8 enters into a complex with FAK and CPN2, engaging a signaling pathway which induces cell migration (Barbero et al., 2009).

In a Drosophila model of tumor invasion, a non-apoptotic effector caspase pathway is utilized to activate the key invasion protein Mmp1 via JNK signaling (Rudrapatna et al., 2013) (Figure 3). It has been proposed that this cell invasion is achieved through co-opting functions of apoptotic caspase such as cytoskeletal modification. This could be extrapolated as a general feature of non-apoptotic caspase activities in different processes. Together, this suggests a new way of thinking about caspases. Perhaps it is more constructive to think of caspases as cell-structure modifying enzymes rather than as just cell death effectors. This idea is consistent with emerging data showing the role of caspases in neuronal plasticity.

NEURAL SIGNALING AND POTENTIATION

During early development, live imaging of caspase activity in the brain shows a complex pattern of expression and subcellular localization, occurring in discrete waves (Oomman et al., 2006). These waves of activity correspond to specific periods of brain maturation. Here, we look at the roles of this non-apoptotic caspase activity in neuronal network pruning, synaptic plasticity, signal modulation, and axonal guidance (Hyman and Yuan, 2012).

FIGURE 3 | Model of caspase-mediated tissue invasion based on Drosophila studies. A sub-apoptotic level of caspase activation leads to the activation, via JNK signaling, of matrix metalloproteases. This metalloprotease activity is a necessary step in the invasion of tissues. Abbreviations: JNK, Jun kinase; Mmp1, Matrix metalloprotease 1; Hid, head involution defective; Dronc, Drosophila Nedd2-like caspase; Drice, Drosophila ICE.
Pruning of axons and dendrites are the mechanisms through which undesired neural connections are removed. Neural network pruning during larval development in Drosophila is carried out through severing the connection between the outgrowth and the cell body, by means of localized executioner caspase activity, which is mediated by the spatially-restricted degradation of IAP proteins through caspase-3-like activity. An essential step in the process is degradation of DIAP1, a key inhibitor of caspase activity. Inhibition of the caspase-3-ortholog Dronc prevents this pruning process (Kuo et al., 2006) (Figure 4).

In mammals, caspases were found to modulate synaptic plasticity through localized activation within synaptic terminals and neurites in response to stressors. Caspase-3 activity leads to dephosphorylation and internalization of AMPA-type receptors. The loss of these receptors causes degradation of the local dendritic spine. This leads to overall modulation of glutamate signaling. Consequentially, caspases have a role in long-term depression (LTD) of neurons, and overexpression of the anti-apoptotic proteins XIAP or Bcl-xL prevent this LTD (Li et al., 2010c).

Axonal guidance is carried out through the diffusion of molecular signals by the target site, generating a chemotrophic gradient for the axon. Caspases also contribute to this chemotrophic migration by regulating the growth of neurites, through localized proteolytic activity within growth cone structures. Caspase-3 activation is required for this response, as LPA-induced growth cone collapse and netrin-1-induced growth cone attraction are both blocked by caspase-3 inhibitors (Campbell and Holt, 2003). This caspase-3-mediated effect does not require caspase-9 activation, suggesting a distinct, non-canonical activation pathway. It has been speculated that caspase-3 mediated modulation of growth cones is carried out through degradation of cytoskeletal structural elements such as actin and gelsolin (Campbell and Holt, 2003) and rock-1 (Riento and Ridley, 2003).

Such caspase-mediated modulation of synaptic plasticity, axon pruning, and growth cone mobility appear to be cell-autonomous events, in that all utilize localized caspase activity within the target cell, likely through spatially-restricted degradation of inhibitors of caspase activity.

CONCLUSION

Here, we reviewed the major non-apoptotic roles of caspases discovered to date. We discussed such roles in terms of different cell behaviors such as differentiation, migration, and cell signaling, and presented evidence for the cell autonomous and non-cell-autonomous models of caspase signaling.

In some systems, it seems rather clear that a cell autonomous event is occurring. This is the case in, for example, axonal pruning, where a defined cell autonomous pathway of caspase activation has been elucidated. Other systems appear to be examples of the non-cell-autonomous model. An example of this is the process of compensatory proliferation, whereby caspase-generated signals from apoptotic cells stimulate the proliferation of nearby cells in an intercellular, receptor mediated fashion. Finally, there are systems where the evidence is conflicting. This includes the process of myoblast differentiation, in which there appears to be an essential role for both cell membrane contact with apoptotic, PS exposing cells, and for the cell autonomous caspase activation of nucleases to enable the transcription of myogenic genes. Some further approaches that may prove fruitful for this field include the live imaging of caspase activity in individual cells undergoing differentiation, the identification of soluble mitogenic signaling factors from apoptotic cells, and investigation of the interplay between caspase signaling pathways and other signaling pathways.

Regardless of how caspases are regulated in these models, it seems clear that caspases do indeed have roles other than as effectors of cell death. This new understanding suggests unexpected complications in situations where caspase-dependent cell death is considered desirable, such as in response to cancer chemotherapy. The newfound alternative roles of caspases present the possibility that chemotherapy drugs may induce a wide range of cell behaviors such as increased migration and compensatory proliferation of cancer cells (Jäger and Zwacka, 2010) that are both unexpected and unwanted.

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Connolly et al. New roles for killer caspases
The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 17 December 2013; accepted: 28 March 2014; published online: 16 April 2014.

Citation: Connolly PF, Jäger R and Fearhead HO (2014) New roles for old enzymes: killer caspases as the engine of cell behavior changes. Front. Physiol. 5:149. doi: 10.3389/fphys.2014.00149

This article was submitted to Striated Muscle Physiology, a section of the journal Frontiers in Physiology.

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DNA-PK activity is associated with caspase-dependent myogenic differentiation

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Keywords
apoptosis; caspase-3; differentiation; DNA-damage; DNA-PK; muscle; myogenesis

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(Received 26 January 2016, revised 27 July 2016, accepted 10 August 2016)
doi:10.1111/febs.13832

Differentiation of myoblasts into myotubes is essential for skeletal muscle development and regeneration. Caspase-3 and caspase-9 are required for efficient myoblast differentiation. The caspase-activated endonuclease activity, CAD, and the DNA-damage repair protein XRCC1 have also been shown to be required to complete differentiation. DNA-damage associated with differentiation is accompanied by phosphorylation of Histone 2AX, an event normally catalysed by kinases ATR, ATM or DNA-PK. However, the kinase responsible for phosphorylation during differentiation is not known. Here we show that inhibition of DNA-PK, but not of ATR or ATM, blocked histone phosphorylation during differentiation. We also show that DNA-PK inhibition and siRNA-mediated DNA-PK knockdown blocked cell fusion. These data implicate a new role for DNA-PK in myogenic differentiation.

Introduction

Caspases have historically been associated with the process apoptotic cell death. Due to their very wide substrate range and their ability to autoactivate, it had long been assumed that caspase activation was inherently destructive, acting as a point of no return in apoptotic cell-death. However, it has become clear that caspases serve roles other than apoptotic cell death. These roles include the facilitation of cell motility and cytoskeletal reorganization, the generation and transmission of signalling molecules, and the differentiation of precursor cells [1].

Myogenic satellite cells (myoblasts) are a population of precursor cells which are found at the periphery of muscle fibres and serve as a regenerative reservoir to facilitate repair and development. Myoblast differentiation has previously been shown to require the activity of caspases, including caspase-3 and -9 [2,3] and can be blocked by overexpression of Bel-XL, a key anti-apoptotic gene [3]. Caspase-dependent myoblast differentiation also requires the induction of DNA damage through activation of an endonuclease, CAD [4]. This damage is accompanied by the appearance of phosphorylated histone H2AX (γH2AX), a marker of DNA damage [4]. These and other data [5] implicate not just apoptotic caspases in differentiation, but also other key components of the apoptotic process.

Recently, XRCC1, a protein involved in the DNA damage response (DDR) has also been shown to play a role in caspase-3 dependent myogenic differentiation

Abbreviations
ATM, ataxia telangiectasia mutated; ATR, ATM- and RAD3-related; CAD, caspase activated DNase; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate; DEVD-AMC, N-acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin; DMEM, Dulbecco’s modified eagle medium; DNA-PK, DNA-dependent protein kinase catalytic subunit; DTT, dithiothreitol; EGTA, ethylene glycol-bis[β-aminoethyl ether]-N,N,N’,N’-tetraacetic acid; FBS, fetal bovine serum; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MOMP, mitochondrial outer membrane permeabilization; NHEJ, non-homologous end joining; PARP1, poly(ADP-ribose) polymerase 1; SCID, severe combined immunodeficiency; XRCC1, X-ray repair cross-complementing protein 1.
XRCC1 plays a role in repair of single strand breaks in and microhomology-mediated end-joining repair of double strand breaks. During differentiation XRCC1 is recruited to DNA breaks in the p21 promoter, mediating repair and increasing the expression of p21, expression that is important for muscle regeneration in vivo [7].

The phosphorylation of H2AX implicates a role for nuclear PI-3 kinases in myoblast differentiation. The PI-3 related kinase superfamily includes several DNA-damage response factors, such as Ataxia telangiectasia mutated (ATM), ATM- and RAD3-related (ATR), and DNA-dependent protein kinase catalytic subunit (DNA-PK) as well as mammalian target of Rapamycin (mTOR) [8].

In a canonical DNA-damage response the phosphorylation of H2AX, is catalysed by members of PI-3 related kinase superfamily Ataxia telangiectasia mutated (ATM), ATM- and RAD3-related (ATR), or DNA-dependent protein kinase catalytic subunit (DNA-PK) [9]. ATM plays a role in BER, a process that also involves XRCC1. ATM and ATR play role in homologous recombination, while DNA-PK is a key enzyme in non-homologous end-joining.

During apoptosis H2AX is phosphorylated by DNA-PK [10]. This phosphorylation is caspase independent but is required for apoptotic DNA fragmentation [11]. This has led to the suggestion that apoptotic DNA fragmentation is the consequence of two parallel pathways, one involving caspase-3 and ICAD cleavage, and the other DNA-PK and H2AX phosphorylation [11].

Here, we set out to identify the kinase responsible for caspase-3 dependent H2AX phosphorylation during myoblast differentiation. To accomplish this, we screened a panel of pharmacological inhibitors of PI3 kinases, including inhibitors of ATM, ATR and DNA-PK to test which, if any, blocked histone phosphorylation during caspase-dependent differentiation and whether any inhibitor blocked caspase-dependent fusion.

Results

Pharmacological inhibition of DNA damage response kinases

The differentiation of C2C12 cells into myotubes involves caspase-dependent phosphorylation of H2AX [4], an event that is required for the repair of damaged DNA [9]. H2AX phosphorylation is catalysed by several DNA damage response (DDR) kinases. To investigate which DDR kinases may be required in caspase-dependent differentiation, we tested a panel of pharmacological inhibitors of various DDR kinases for their effect on H2AX phosphorylation and myogenic cell fusion.

To test the cytotoxicity of the compounds, a cell viability screen was conducted at a range of concentrations (1 nM to 30 µM) of each inhibitor. The effect of each drug on cell viability was tested in both growth medium and differentiation medium. After 72 h in the presence of the inhibitor, viability was assayed by the resazurin cellular reductase assay (Fig. 1A–C). Neither KU-55933 (ATM inhibitor) nor NU-7441 (DNA-PK inhibitor [12]) showed cytotoxicity at 1 µM when cells were cultured in growth medium. However, when cells were cultured in differentiation medium NU-7441 induced a small decrease in cell viability. VE-821 (ATR inhibitor) was cytotoxic at 1 µM irrespective of the culture medium.

NU-7441 and NU-7026 block H2AX phosphorylation during differentiation

Phosphorylation of H2AX is a consequence of myogenic DNA damage [4], but the kinase responsible for this phosphorylation has yet to be determined. Therefore, we tested whether the DNA-PK inhibitors NU-7441 and NU-7026 blocked H2AX phosphorylation during differentiation. This was assessed by scoring the number of nuclei with more than 25 foci using immunofluorescence. A representative micrograph of each condition is presented in Fig. 2A. We first assessed the effect of NU-7441 on the time course of H2AX phosphorylation during differentiation (Fig. 2B). During differentiation, H2AX phosphorylation rises to a peak at 24 h, a timeline that is concomitant with caspase activity and DNA damage [4,6]. This phosphorylation was effectively blocked by the pan-caspase inhibitor Q-VD-OPh; consistent with earlier reports that γH2AX appearance is caspase dependent [4]. The phosphorylation was also blocked by the DNA-PK inhibitor NU-7441.

To look for more subtle effects on H2AX phosphorylation, we enumerated all foci in each nucleus (Fig. 2D). After 24 h in low-serum media, the percentage of nuclei with > 25 foci increased significantly from 18% to 30%, and there was a general shift towards higher numbers of foci per nucleus. This increase in H2AX phosphorylation was blocked by the pan-caspase inhibitor Q-VD-OPh. This increase was also blocked by the DNA-PK inhibitors NU-7441 and NU-7026.

Since ATM and ATR are also known to phosphorylate H2AX, we compared the effect of VE-821 (ATR
inhibitor), KU-55933 (ATM inhibitor), and NU-7441 on H2AX phosphorylation. Unlike NU-7441, the ATR and ATM inhibitors did not affect the proportion of cells that were γH2AX positive (Fig. 2C).

Effect of DDR kinase inhibitors on myogenic fusion

To test whether inhibitors of PI-3-like kinases blocked myogenic fusion, C2C12 cells were treated with VE-821, KU-55933 or NU-7441 (all 1 μM) and myogenic fusion assessed after 72 h. Neither VE-821 nor KU55933 affected cell fusion (Fig. 3A; 28.3% and 30.4% respectively), even though VE-821 was cytotoxic at the concentration used (Fig. 1A). PI3K inhibitors (PI-828 and LY294002) and an ATM inhibitor (GCK-733) (all at 1 μM) also failed to inhibit cell fusion (data not shown). However, the inhibitor of DNA-PK, NU-7441 significantly reduced cell fusion at 1 μM (Fig. 3A,B; fusion index of control = 29.0% and of NU-7441 treated cells = 11.9%).

Concentration-dependent inhibition of cell fusion by NU-7441 and NU-7026

To assess if the effect on myogenic fusion of NU-7441 and DNA-PK inhibitor NU-7026 was concentration dependent, cells were treated at a range of concentrations (10 nM to 3 μM) (Fig. 3C). When fusion was assessed at 72 h, a strong dose-dependent reduction was observed, with the IC₅₀ estimated to be 1 μM (Fig. 3C).

Knock down of DNA-PK blocks cell fusion

To corroborate the pharmacological data, three separate siRNAs specific for DNA-PK were transiently expressed in C2C12 cells and the effect on cell fusion assessed. All three specific siRNAs (50 nM) decreased cell fusion relative to a non-specific control siRNA (Fig. 4A,B).

NU-7441 and NU-7026 do not block caspase activity during differentiation

To test if DNA-PK activity has a role in caspase activation the effect of NU-7441 and NU-7026 on differentiation-induced caspase-3 activity was measured. As expected, differentiation produced a transient increase in caspase-3 activity, with peak activity at 24 h (data not shown). This was abolished by the pan-caspase inhibitor Q-VD-OPh (Fig. 5A). Caspase-3 processing was also examined by immunoblotting. Unprocessed caspase-3 (p32) was detectable in all samples. Processed forms of caspase-3, including p17 and p15, detected at both 24 and 48 h post-serum withdrawal. The caspase inhibitor Q-VD-OPh reduced the formation of the p17 and p15 forms but caused the
Fig. 2. (A) Testing the effect of NU-7441 and NU-7026 on H2AX phosphorylation. Cells were induced to differentiate and the number of γH2AX foci per nucleus was assessed at 24 h. (B) The percentage of cells with > 25 γH2AX foci after treatment with NU-7441 (○, 1 μM), Q-VD-OPh (□, 30 μM) or 0.1% DMSO (○). (C) Testing the effect of other DDR kinase inhibitors on H2AX phosphorylation. Cells were induced to differentiate in the presence of 1 μM VE-821, KU-55933 or NU-7441. At 24 h the number of nuclei with > 25 γH2AX foci was assessed. 300 nuclei were scored per group per experiment. The data are reported as the fold change (log2) relative to an untreated control (n = 3, ±SEM) (Control 15.0% ± 2.5 and NU-7441 = 3.1% ± 0.3). (D) The effect of NU-7441 and NU-7026 on the percentage of nuclei with γH2AX foci. *P < 0.05, ANOVA and Dunnett’s post-hoc test.
appearance of a ~p20 subunit. These data suggest that Q-VD-OPh inhibited the autocatalytic maturation of caspase-3 to p17 and p15 but did not block processing of full-length caspase-3 by the initiator caspase-9. Treatment of cells with NU-7441 at a concentration that blocked differentiation (1 μM) did not block caspase activity or caspase processing (Fig. 5A,B). Together, these data indicate that NU-7441 does not exert its inhibitory effect on fusion through inhibition of caspase processing or activity.

**NU-7441 does not alter differentiation-associated DNA damage**

Since DNA damage is a necessary event during differentiation [4], we tested whether NU-7441 affected DNA damage using the comet assay. 24 h after differentiation was induced the number of cells with tails > 9 μm was increased compared to a culture that was not induced to differentiate (Fig. 6A,B). This is consistent with earlier reports [4,6]. Treatment of cells with NU-7441 (1 μM) had no effect on the length of the comet tails at 24 h post-serum withdrawal (Fig. 6A) when inhibition of damage repair would be expected to increase the level of DNA damage detected. We have no direct evidence to explain this discrepancy. However it is possible that DNA-PK inhibition causes a lethal accumulation of DNA damage in some cells that die and are lost to our analysis. Indeed, NU-7441 treatment of differentiating cells induced a small but statistically significant decrease in the number of adherent cells (Fig. 6C) that is also consistent with the differentiation-specific decrease in cell viability seen earlier (Fig. 1C).

**Discussion**

Caspase activity is reported as inducing expression of muscle specific genes and myoblast fusion [2,4]. In our
hands the inhibiting caspase activity blocks fusion but has only a minor effect on the expression of muscle specific genes [3]. Differentiation in myoblasts is also associated with a caspase-dependent increase in H2AX phosphorylation. Here we identified DNA-PK as the kinase responsible for this phosphorylation and also show that the kinase was important for differentiation.

Three kinases are known to phosphorylate H2AX: DNA-PK, ATM and ATR. Pharmacological inhibition of DNA-PK, but not ATM or ATR, blocked the H2AX phosphorylation associated with differentiation. Moreover, two pharmacological inhibitors of DNA-PK activity and caspase-dependent differentiation

Fig. 4. Testing the effect of DNA-PK siRNAs on cell fusion. (A) Cells were transiently transfected with non-specific siRNAs (Control siRNA) or three different specific siRNAs (siRNA1-3) to DNA-PK (PRKDC). After 5 days cell fusion was assessed. 300 nuclei were scored per group per experiment ($n = 3$, ±SEM). (B) Representative immunofluorescence images. (C) After siRNA treatment, PRKDC mRNA levels were assessed by RT-PCR and agarose gel electrophoresis (a representative image).

Fig. 5. Testing the effect of NU-7441 on caspase activation. Cells were induced to differentiate in the presence or absence of NU-7441 or Q-VD-OPh. (A) Caspase-3 like activity was measured after 24, 48 and 72 h. The data are expressed as fold activation of the caspase activity in undifferentiated cells (0 h in DM) ($n = 3$, ±SEM) *$P < 0.05$, ANOVA and Dunnett’s post-hoc test. (B) Caspase-3 processing at the indicated times was detected by immunoblotting. The proform and active forms of caspase-3 (p20, 17 and 15) are indicated. ★ indicates a p28 form generated by autocatalytic cleavage at Asp28.
PK blocked myoblast differentiation, while ATM and ATR inhibitors did not.

Inhibition of myogenic differentiation was concentration-dependent with EC$_{50}$ values of approximately 1 µM for both NU-7441 and NU-7026. The reported IC$_{50}$ against DNA-PK in cell-free assays is 14 and 230 nM. The other reported targets for NU-7441 all have IC$_{50}$ values $\geq$ 5 µM. The difference between the EC$_{50}$ and IC$_{50}$ values for NU-7441 may be explained by the need for the inhibitors to penetrate the cell, competition with intracellular ATP and the abundance of DNA-PK [13]. The role of DNA-PK was corroborated using three independent siRNAs against DNA-PK, all of which inhibited myoblast differentiation.

The DNA-PK inhibitors and siRNA against DNA-PK produced a defect in myoblast differentiation very similar to that seen after inhibition of caspase-3 activity, overexpression of the anti-apoptotic protein Bcl-X$_L$ or caspase-9 knock-down [3]. A similar differentiation defect is also seen when cells lose expression of either CAD [4] or XRCC1 [6]. Together these data suggest a model in which a caspase-generated DNA-damage response is important for myoblast differentiation. In this model caspase-3 activates CAD which causes DNA damage. This damage leads to H2AX phosphorylation by DNA-PK. At this time we cannot differentiate between caspase-3 and DNA-PK functioning in the same pathway or in parallel pathways, both required for differentiation.

Although DNA-PK’s best understood role is in non-homologous end joining following double-stranded DNA breaks, DNA-PK can directly interact with XRCC1 [14] and phosphorylate XRCC1 following dsDNA breaks [15]. It also recruits PARP1 to XRCC1 at the ssDNA of a stalled replication fork [16]. The dual role for DNA-PK and XRCC1 in differentiation suggests a repair complex similar to that formed at a stalled fork is generated by CAD-mediated DNA breaks.

In the most recent model proposed by Larsen et al. and Al-Khalaf et al., the DNA-damage is a key event in turning on muscle specific genes. DNA-PK has previously been shown to play a role in regulating gene expression that involves DNA-damage [17]. Insulin
induced changes in gene expression involve DNA damage mediated by topoisomerase IIβ, and blocking DNA-PK reduces this insulin-dependent DNA-damage [17]. Our data are compatible with DNA-PK playing a role in controlling transcription during differentiation. NU-7441 did not increase DNA damage during differentiation, which would be expected if DNA-PK-mediated repair were part of the differentiation process. These data apparently uncouple DNA-mediated repair from the differentiation. However we cannot exclude the possibility that blocking DNA-PK does increase DNA damage but that cells with this increased level die and are lost to our analysis. Indeed, NU-7441 caused a slight decrease in cell viability and cell number when cells were cultured in differentiation medium (Figs 1C and 6C). This could be explained by cell-death that follows an inability to repair DNA-damage. These two models are not incompatible and it may be that the DNA damage repair machinery both turns on genes required for differentiation and at the same time rescues differentiating cells from otherwise lethal DNA damage.

Muscle development is defective in XRCC1 null animals [6] and muscle regeneration is compromised in p21 null mice [7] and mice expressing a caspase inhibitor, p35 [18]. But does DNA-PK play an in vivo role in muscle development or regeneration? Muscle regeneration in SCID mice (which contain a nonsense mutation in DNA-PK) is normal [19] suggesting that the answer is no. The caveat is that the phenotype of SCID mice and mice lacking DNA-PK is not the same [20]. The mutant SCID DNA-PK retains a kinase domain and low levels of the protein are detectable in SCID cell lines [21–24]. Thus, while it cannot take part in NHEJ, the mutant DNA-PK could in principle play a role in other processes, an idea that needs to be tested in vivo.

In conclusion, we have shown that DNA-PK activity is important for myoblast differentiation. Both caspase-3 and DNA-PK activity were required for H2AX phosphorylation during differentiation, a situation that closely resembles the events in apoptosis. Taken with earlier reports that caspase-9 is required for myoblast differentiation and that this differentiation is blocked by overexpression of Bcl-XL [3], it appears that the pathways activated in myoblast differentiation and apoptosis are very similar, if not identical. Nonetheless, the mechanism that allows the same process to induce two such different fates remains obscure. These data also contribute to the ongoing re-evaluation of previously accepted “points of no return” in cell death. It is now clear that neither MOMP [25], caspase-3 or CAD-induced DNA damage necessarily commit a cell to die and the lines demarcating initiation of cell death processes and their execution continue to blur.

Materials and methods

C2C12 myoblast cell line culture and differentiation

Mouse C2C12 myoblasts (Sigma-Aldrich, St. Louis, MO, USA) were seeded at $5 \times 10^5$ cells cm$^{-2}$ and incubated at 37 °C in 5% CO$_2$ atmosphere for 24 h in DMEM supplemented with 10% fetal bovine serum (FBS) and Penicillin/Streptomycin (Growth Medium; GM). To induce differentiation, cells were washed three times with Hank’s Balanced Salt Solution (HBSS) and cultured in DMEM supplemented with 2% horse serum and Penicillin/Streptomycin (Differentiation Medium; DM).

Quantitation of myotube formation by myosin heavy chain immunofluorescence

Cells differentiated on tissue culture plates or poly-D-lysine-treated glass coverslips were washed to remove non-adherent cells and fixed in 4% paraformaldehyde at room temperature for 20 min. Cells were permeabilized (0.1% Triton X-100) and blocked with 10% FBS before being stained with anti-Myosin Heavy Chain antibody (MF-20; Developmental Studies Hybridoma Bank, Iowa City, IA, USA; 1 : 100) and detected with a secondary goat anti-mouse antibody conjugated to Alexa Fluor 488 (Molecular Probes, Eugene, OR, USA; 1 : 1000). Nuclei were counterstained with Hoechst 33342 (2 $\mu$g mL$^{-1}$). Myotubes were visualized under inverted epifluorescence microscopy and images captured using a CCD. The extent of fusion was quantified using the fusion index, where the number of nuclei found within myotubes (defined as syncytia containing two or more nuclei) was expressed as a percentage of the total number of nuclei per field.

Drug treatments

Cells were seeded in 96 well plates and cultured in DM or GM supplemented with drugs or vehicle control (0.1% DMSO) for the duration of the experiment. The inhibitors used were Q-VD-OPh hydrate (Apexbio, Houston, TX, USA), VE-821, NU-7441 and KU-55933 (Tocris Biosciences, Bristol, UK).

Resazurin cell viability assay

Cells were grown and differentiated in 96 well plates. At the endpoint, the differentiation medium was removed and replaced with fresh DM. This was supplemented with resazurin dye (Sigma-Aldrich) to a final concentration of
93 μM. The plates were incubated for 6 h at 37 °C in 5% CO₂. Dye reduction was measured using a Victor 3V 1420 spectrometer (λex 530 nm, λem 595 nm).

Caspase-3-like activity assay

Adherent cells were harvested by trypsinization and centrifuged at 400 g for 5 min, before being resuspended in lysis buffer (50 mM HEPES, pH 7.4, 10 mM KCl, 2 mM MgCl₂, 5 mM EGTA, 0.1% CHAPS, 100 mM PMSF, and 1 mM DTT). Protein concentrations were determined by Bradford assay and then normalized. A fluorogenic caspase-3 substrate DEVD-AMC (Sigma-Aldrich) was added to a concentration of 10 μM and changes in fluorescence (λex 380 nm and λem 450 nm) over time detected using a Perceptive Biosystems CytoFluor Series 4000 fluorimeter. The rate of enzymatic activity was determined from the linear phase of the time-course, and was reported as AFU·min⁻¹·mg⁻¹ total protein.

Single cell gel electrophoresis (comet) assay

Cells were harvested, combined with low-melting point agarose and pipetted onto a microscope slide. The slide was immersed for 30 min in chilled lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris base, 1% N-laurylsarcosine, and 0.5% Triton X-100). The slides were then transferred to chilled alkaline solution (0.3 M NaOH, 10 mM EDTA) and incubated for 20 min. After this, the slides were subjected to electrophoresis at 35 V (4.3 V/cm⁻¹) for 20 min. After ethanol fixation DNA was stained with SYBR Green and images were captured. The percentage of cells showing DNA damage and the length of each comet tail was scored.

Immunofluorescence for γH2AX

Cells were washed twice with PBS and pre-extracted with 0.5% Triton X-100 in PBS with 100 μg·mL⁻¹ RNase A for 30 min at room temperature. Cells were fixed in 4% paraformaldehyde in PBS for 15 min at room temperature. Cells were then permeabilized with 0.2% Triton X-100 for 5 min at room temperature. The coverslips were blocked with 10% FBS and 2% BSA in PBS and stained with an antibody against γH2AX (1 : 50 dilution; Cell Signaling Technology, Danvers, MA, USA) and a Alexa Fluor 488 or 594 secondary antibody (1 : 1000; #9662, Cell Signaling Technology). The cells were counterstained with Hoechst 33342 at 2 μg·mL⁻¹, before being visualized using inverted epifluorescence microscopy. Images were recorded using a CCD.

Preparing whole cell lysates for immunoblotting

Cell monolayers were trypsinized and collected by centrifugation. The cells were resuspended in RIPA lysis buffer supplemented with protease and phosphatase inhibitor cocktails (Sigma-Aldrich). The suspensions were vortexed vigorously and an aliquot used to determine the protein concentration. The remaining lysate was mixed with 2 x SDS/PAGE loading buffer and heated at 90 °C for 5 min.

Immunoblotting

Proteins resolved by SDS/PAGE were transferred to nitrocellulose membranes. Membranes were blocked with 5% non-fat milk and probed with a primary anti-caspase-3 antibody (1 : 1000; #9662, Cell Signaling Technology). IRDye 800CW or 680RD secondary antibodies (1 : 10 000 dilution) were used to visualize proteins using an Odyssey CLx point-source laser scanner (Liczor Biosciences, Lincoln, NE, USA).

siRNA reverse transfection

Polyethylenimine (PEI) from Polysciences (Cat 23966) was prepared in water (1 mg·mL⁻¹), filtered (0.2 μm) and stored at −20 °C until used. Stock solutions (20 μM) of siRNAs from Origene were prepared as directed by manufacturer. 30 pmoL siRNA was added to 28.5 μL PEI was added to 27.9 μL NaCl (0.15 M). PEI (2.1 μL) was added to 27.9 μL NaCl. The siRNA and PEI solutions were mixed and incubated at room temperature for 30 min. 20 μL of this reaction mix was aliquoted into a well on a 96 well plate and 180 μL of C2C12 cell suspension (4.4 x 10⁴ cells·mL⁻¹ in GM) was added. After 24 h the cells were washed cells and add fresh medium added. After 48 h the GM was replaced with DM.

Reduced expression of DNA-PK was tested by RT-PCT using the following primers: PRKDC F 5′-AAGGCAG AAGCCTGGACAAATG 3′; PRKDC R 5′-ATCCGCC AGTAGGTCAATGCTG 3′; GAPDH F 5′-CCCCAA TGTTGCTCGTGTG 3′; GAPDH R 5′-GCCGCTTCC ACCACCTTCT 3′.

Data analysis and statistics

All numerical datasets with greater than two groups were first tested for normality with D’Agostino’s K-squared test. Following this, one-way ANOVA was used to test for statistical significance within the dataset, and Dunnett’s post-hoc test was used to compute mean differences and assess statistical significance. All datasets with two groups were subject to Student’s unpaired t-test.

Acknowledgements

We thank Peter Vandenabeele for constructive criticism. This research was supported by a College of Medicine Postgraduate Scholarship to PFC.
Author contributions

PFC and HOF planned experiments; PFC performed experiments; HOF and PFC analysed data; HOF and PFC wrote the paper.

References

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