Stress-sensing in the human greying hair follicle: Ataxia Telangiectasia Mutated (ATM) depletion in hair bulb melanocytes in canities-prone scalp.

*Stephen K. Sikkink[1], Solene Mine[2], Olga Freis[2], Louis Danoux[2], & *Desmond J. Tobin[1,3]

[1]Centre for Skin Sciences, School of Life Sciences, University of Bradford, UK. [2]BASF Beauty Care Solutions France S.A.S., Pulnoy, France. [3]The Charles Institute of Dermatology, School of Medicine, University College Dublin, Dublin, Ireland.

*Corresponding Authors

Dr. Stephen Sikkink. Centre for Skin Sciences, School of Life Sciences, University of Bradford, Richmond Rd. Bradford. West Yorkshire. BD7 1DP, UK. Correspondence by email to s.k.sikkink@bradford.ac.uk

Prof. Desmond J Tobin, The Charles Institute of Dermatology, University College Dublin, Dublin 4, Ireland. Correspondence by email to desmond.tobin@ucd.ie

Supplementary Table 1. Sources of antibodies used for Immunohistochemistry. All primary antibodies were used at a 1:100 dilution for immunohistochemistry apart from GP100 which was used at a 1:50 dilution.

Melanocyte Markers	
Tyrosinase (TYR)	Abcam Mouse Monoclonal #Ab738
GP100 (NKI/beteb)	Monosan Mouse Monoclonal #MON7006-1
Tyrosinase related protein-1 (TRP-1)	Santa Cruz Rabbit Polyclonal #sc-25543 (H-90)
Dopachrome tautomerase (TRP-2)	Santa Cruz Rabbit Polyclonal #sc25544 (H-150)
Apoptosis Markers	
Caspase-2 (CASP-2)	BD Biosciences Monoclonal #611023 (ICH-1L)
Caspase-3 (CASP-3)	Abcam Rabbit Monoclonal #Ab32042
Caspase-9 (CASP-9)	Abcam Rabbit Polyclonal #Ab63342
BCL-2	Santa Cruz Mouse Monoclonal #Sc-509
Cell Cycle/Senescence Markers	
ATM	Abcam Rabbit Monoclonal #Ab32420
p21	Abcam Rabbit Polyclonal #Ab18209
p38	CST Rabbit Polyclonal #9212
phospho-p38 (Thr180/Tyr182)	CST Rabbit Polyclonal #9211
p27	CST Rabbit Polyclonal #3686
phospho-p27 (Ser10)	Santa Cruz Rabbit Polyclonal #Sc-12939
p57	CST Rabbit Polyclonal #2557
phospho-p57 (Thr310)	CST Rabbit Polyclonal #2558
p53	Abcam Mouse monoclonal #Ab1101
phospho-p53 (Ser15)	Abcam Rabbit Polyclonal #Ab1431
DNA Repair Markers	
GADD45	Santa Cruz Rabbit Polyclonal #sc-797
8-OHdG	Acris Mouse Monoclonal #BM2551
Anti-oxidant Enzymes	
Catalase	Sigma Mouse Monoclonal #C0979
Heme oxygenase-1 (HO-1)	Abcam Rabbit Monoclonal #Ab52947
Superoxide dismutase (SOD-1)	Abcam Rabbit Polyclonal #Ab13498
Glutathione peroxidase (GP-1)	Abcam Rabbit Polyclonal #Ab22604
Glutathione reductase (GR-1)	Abcam Rabbit Polyclonal #Ab16801

Supplementary figure 1. Tyrosinase staining is markedly reduced in greying hair follicles compared to GP100 and TRP-1.

Double IF of scalp tissue with TRP-1 (green channel) & TYR (red channel) reveals a decrease in melanocyte labelling from fully pigmented to grey hair follicles. TRP-1 staining was again proportional to the melanin content observed in brightfield images (left panels; hair follicle outline is indicated with black dashed line), however by comparison TYR staining was markedly reduced even in heavily pigmented follicles. Circles in lower panels show TRP-1 negative/TYR positive staining of the last remaining pigmented melanocyte (which is still pigmented in the brightfield image) in a grey hair bulb with near complete (fully grey) canities. DAPI nuclear stain is shown in blue channel. Scale bar = 20 μm. Donor age is indicated on the right.

Supplementary figure 2. Expression of ATM in epidermis and dermis.

Overlays of ATM (green channel) and melanocyte marker GP100 (red channel) in two cases of adult human scalp showing examples of strong nuclear ATM expression in epidermal melanocytes. Less frequent nuclear ATM staining was occasionally detected in isolated differentiating keratinocytes within the basal layer. Similar levels of nuclear ATM staining were not detected in dermal fibroblasts, although some cytoplasmic staining was visible. Right panels show increased magnification (×2) of the epidermis. Yellow arrows indicate double-labelled melanocytes. DAPI nuclear stain is shown in blue channel. Scale bar is shown on upper images. Donor age is indicated on the right.

Supplementary figure 3a. Caspase 2 staining in human hair follicles.

CASP-2 (green channel) expression in two grey hair follicles from a 40-year old normal healthy female donor. Lower panels show expression in the lower IRS and connective tissue sheath in the proximal hair follicle bulb. Upper panels show CASP-2 expression in the IRS/pre-cortex in the upper hair follicle. DAPI nuclear stain is shown in blue channel. Scale bar = 20 μm.

Supplementary figure 3b. Caspase-9 staining in human hair follicles.

CASP-9 (green channel) expression in the bulbs of two pigmented (upper left panels) and two greying (upper right panels) hair follicles showing particularly high expression within the IRS which extended up the follicle (lower two panels). Staining was also occasionally detected within the matrix keratinocytes (white circle). Melanocytes labelled with GP100 are shown in the red channel. DAPI nuclear stain is shown in blue channel. Scale bar = 20 μm. Donor age is indicated next to each image.

Supplementary figure 3c. GADD45 staining in human hair follicles.

GADD45 (green channel) expression was detected in the early differentiating IRS and in the dermal papilla in hair follicles from different donors. Melanocytes labelled with GP100 are shown in the red channel. DAPI nuclear stain is shown in blue channel. Scale bar = 20 μm. Donor age is indicated next to each image.

Supplementary figure 3d. p21 staining in human hair follicles.

Left panel shows striking p21 expression (green channel) in terminally differentiating IRS and dermal papilla (DP) basement membrane from a 23-year old pigmented hair follicle. In comparison the right panel shows less intense IRS p21 expression with additional DP staining in an older 52-year old female. Melanocytes labelled with GP100 are shown in the red channel. DAPI nuclear stain is shown in blue channel. Scale bar = 20 μm.

Supplementary figure 3e. Infrequent p57 and phospho-p57 staining in human hair follicles.

p57 (left upper and lower panels) and phospho-p57 (right upper and lower panels) staining (green channel) was only detected in hair follicles from a 40-year old normal healthy female donor. p57 staining was seen within a distinct region of the ORS in a grey follicle, with expression terminating in the ORS just above the bulb region, whereas phospho-p57 expression was seen in the IRS and extended up towards the bulge region. DAPI nuclear stain is shown in blue channel. Scale bar = 20 μm.

Supplementary figure 3f. GR-1 staining in human hair follicles

GR-1 expression (green channel) was detected specifically in the ORS of multiple hair follicles (pigmented hair follicles from four different donors are shown). Melanocytes labelled with GP100 are shown in the red channel. DAPI nuclear stain is shown in blue channel. Scale bar = 20 µm.

Supplementary figures 3g & 3h. HO-1 staining in human hair follicles

Upper panels (supplementary figure 3g) show HO-1 expression (green channel) in the IRS with specific involvement of a small subset of cells within the hair follicle where IRS cornification is advanced to complete (white arrows) in hair follicles from four donors. Lower panels (supplementary figure 3h) displays two images from a transversely-(serial) sectioned scalp sample from a 50-year old female donor. Strong HO-1 cytoplasmic expression is seen in the IRS cells around the hair fibre (HF). HO-1 expression was also detected in the sebaceous gland (SG). GP100 expression of melanocytes is shown in pigmented hair follicles (red channel). DAPI nuclear stain is shown in blue channel. Scale bar is shown on images. Donor age is indicated next to each image.

Supplementary figure 4. Catalase expression is increased in HFMs after exposure to hydrogen peroxide (uncropped Western blots).

Full uncropped Western blots from figure 4d. Upper image shows catalase protein expression in HFM's after treatment for 1 h with 40 µM hydrogen peroxide. CML = control untreated HFM lysate used to fill empty well. M= molecular weight marker. The catalase blot was stripped and reprobed for GAPDH (lower image) as a loading control.
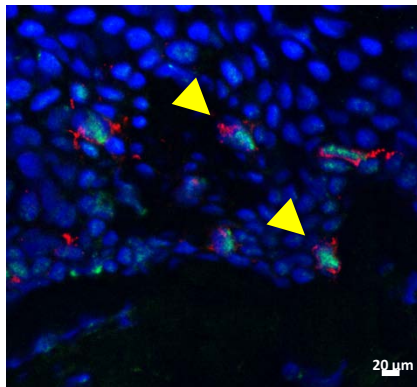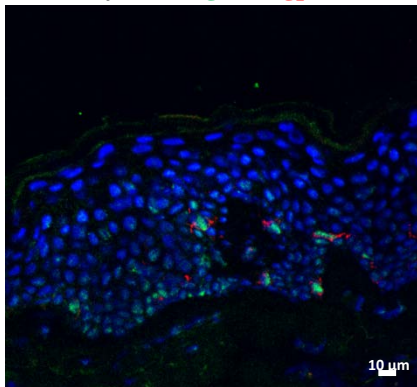
Supplementary figure 5. Total ATM protein expression is reduced by KU60019 in HFM.

Melanocytes were treated with the ATM specific kinase inhibitor KU60019 (5 µM) in triplicate for 24 h then analysed for ATM protein expression by Western blot. Representative Western blot showing reduction of ATM protein expression in KU60019 treated samples
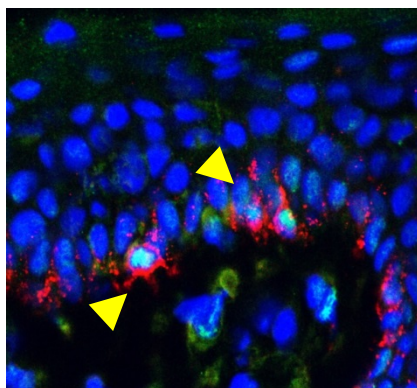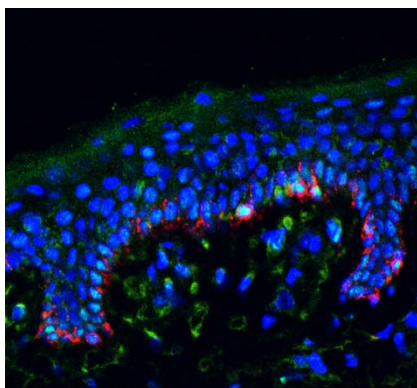
compared to vehicle controls (not significant by t-test). GAPDH (36 kD) was used as a loading control. Graph shows quantification of mean ATM expression (normalised to GAPDH in relative densitometry units) from Western blots. Error bars show SEM. Uncropped images of blots and size markers are also shown.

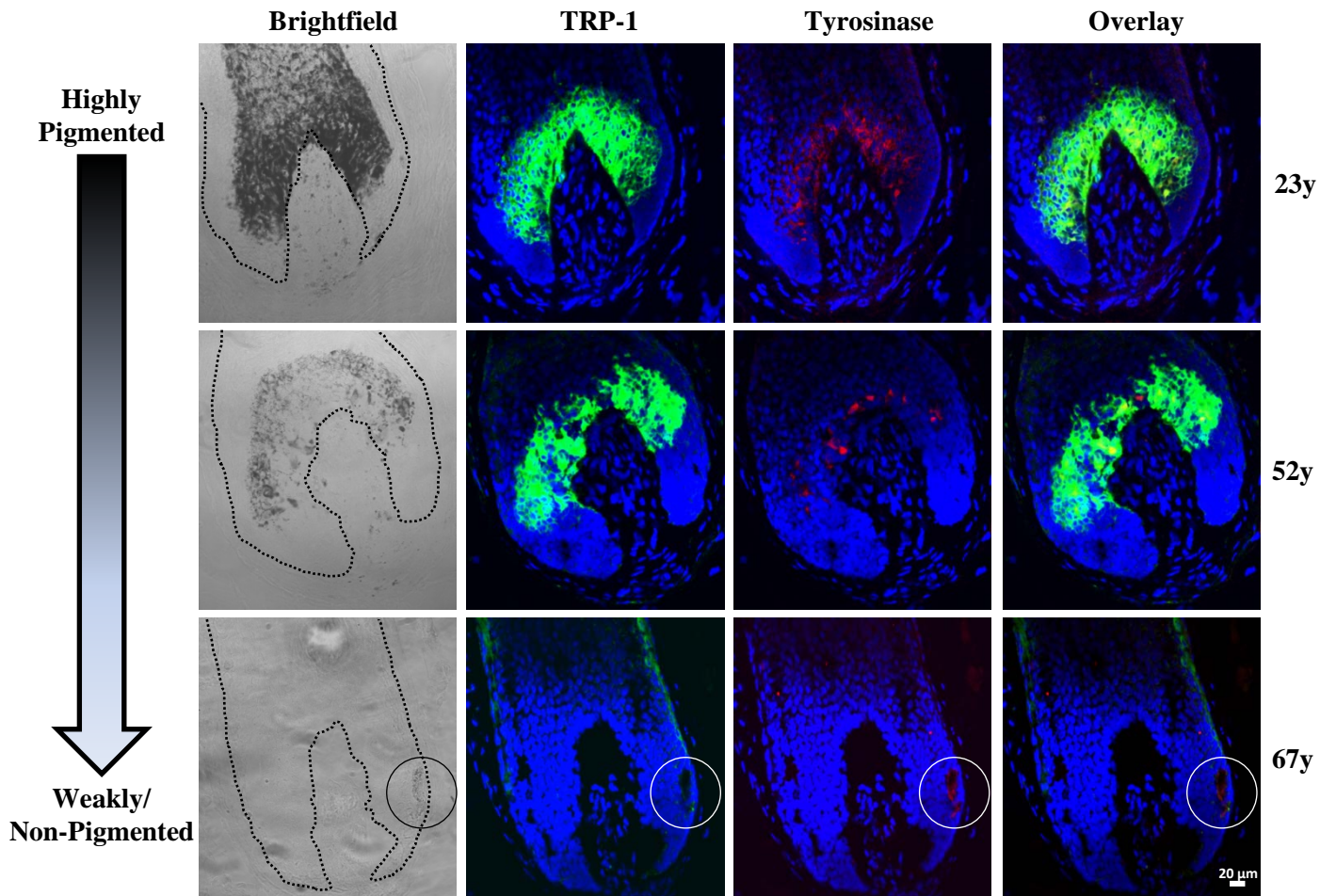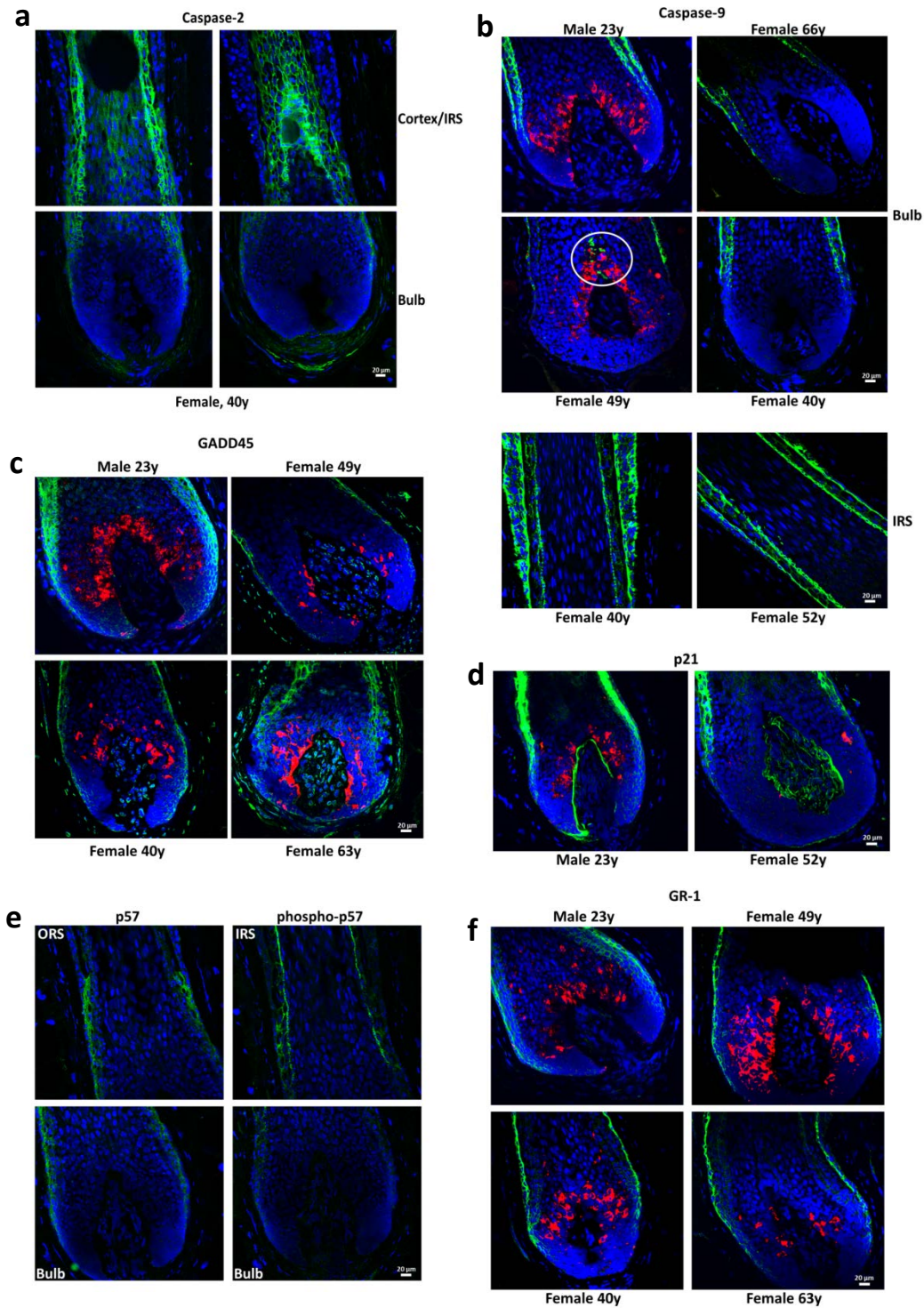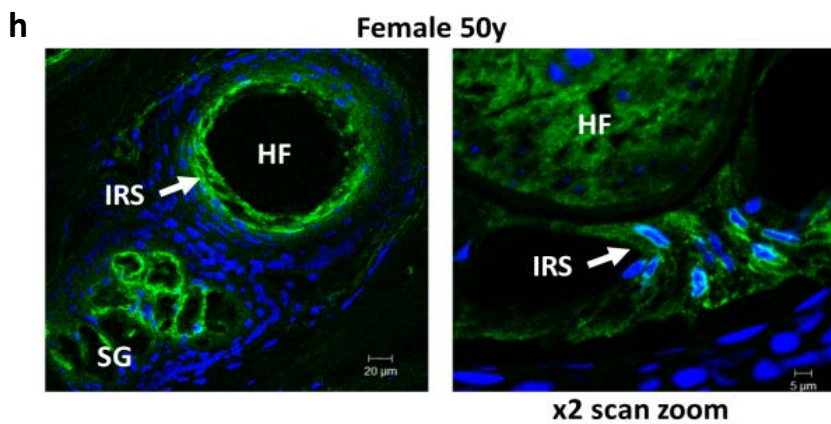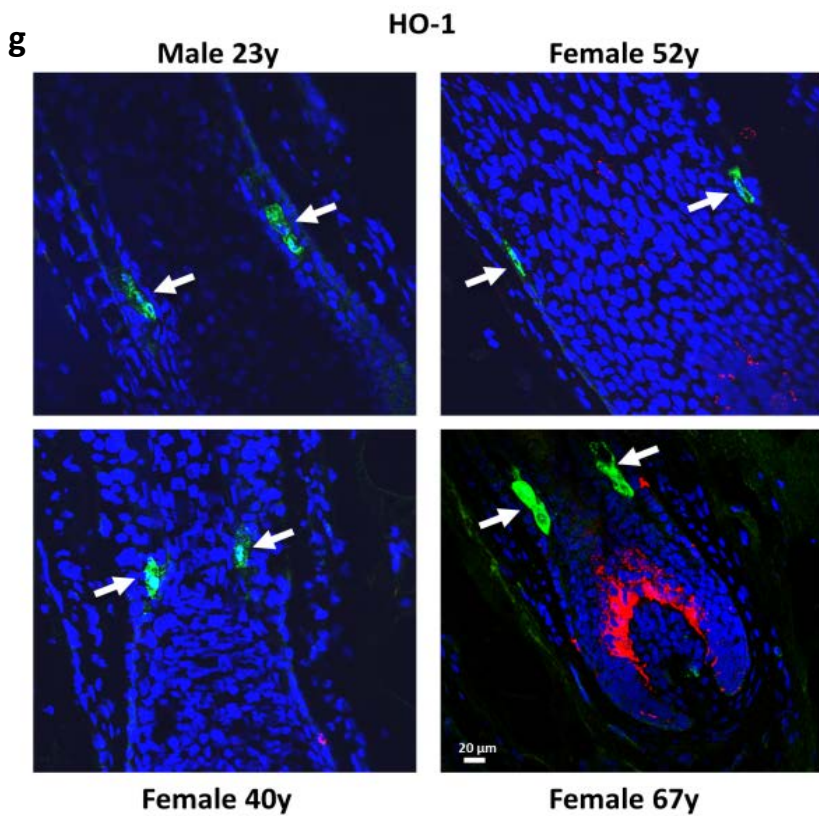| Overlay; ATM-green, gp100-red	Scan Zoom (x2)	

10 µm 20 µm

60y

63y

	Brightfield	TRP-1	Tyrosinase	Overlay

Highly Pigmented

Weakly/ Non-Pigmented

23y

52y

67y

20 µm

a Caspase-2

Cortex/IRS

Bulb

Female, 40y

b Caspase-9

Male 23y   Female 66y

Bulb

Female 49y   Female 40y

IRS

Female 40y   Female 52y

c GADD45

Male 23y   Female 49y

Female 40y   Female 63y

d p21

Male 23y   Female 52y

e   p57   phospho-p57

ORS   IRS

Bulb   Bulb

f GR-1

Male 23y   Female 49y

Female 40y   Female 63y

HO-1

g

Male 23y   Female 52y

Female 40y   Female 67y

h   Female 50y

x2 scan zoom
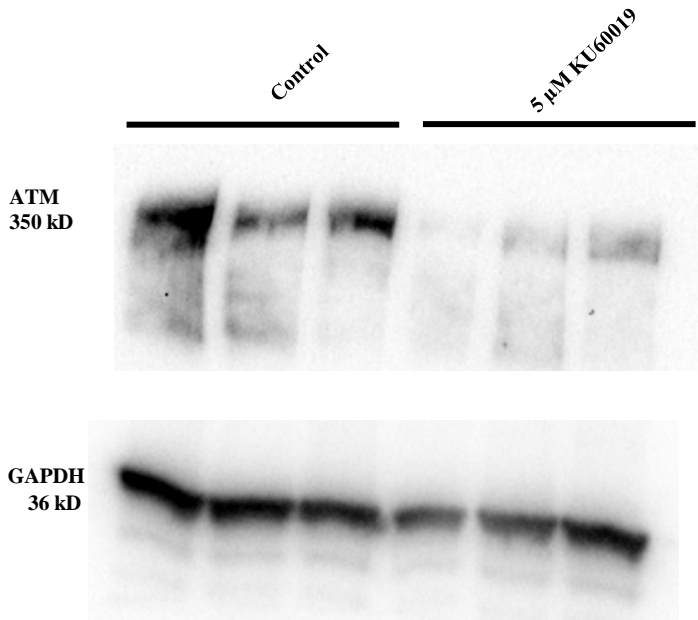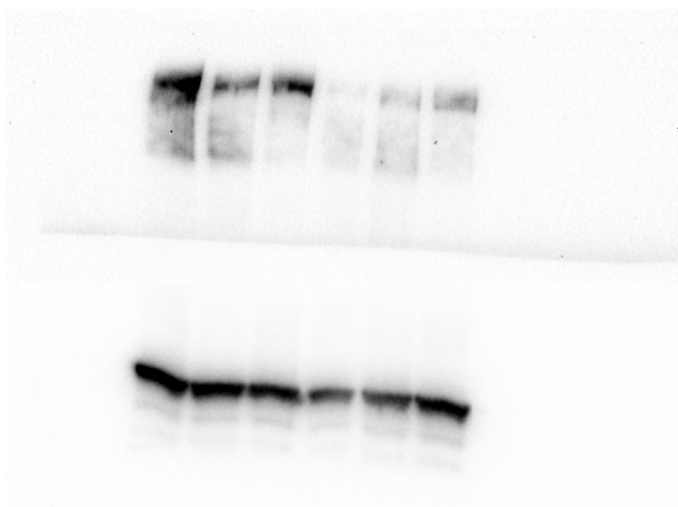
Control   5 µM KU60019

ATM
350 kD

GAPDH
36 kD

Treatment

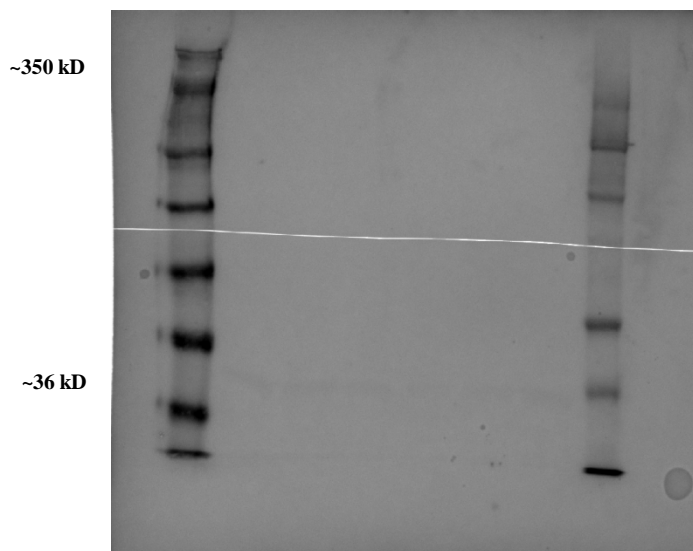# Supplementary Figure 5 Full Western blot

ATM blot

GAPDH blot

~350 kD

~36 kD

Size markers