

Identifyn Standards for Optical Microscopy Methods™Identifyn® Primary Antibody**Rabbit anti-Human ATM.p (Serine 1981) Recombinant Monoclonal, IgG - (BLR031P)***Cat ID# PA-RR 000012***Mouse anti-Human H2Ax Monoclonal, IgG1 - (1H1D9/B7)***Cat ID# - PA-MM 000002*Identifyn® Secondary Antibodies**Identifyn® SRM Alexa Fluor™ 647 Affinity Purified Goat Anti-Rabbit, F(ab')₂ (H + L)***Cat ID# - SA 000063***Identifyn® SRM Alexa Fluor™ 488 Affinity Purified Donkey Anti-Mouse, F(ab')₂ (H + L)***Cat ID# - SA 000019*

ATM (ataxia-telangiectasia mutated) is a crucial serine/threonine kinase that plays a central role in the DNA damage response (DDR), particularly in response to DNA double-strand breaks (DSBs). ATM's activation and subsequent phosphorylation cascades are essential for detecting DNA damage and initiating repair processes. Here's a detailed overview of how ATM is activated, its targets, and its role in the DDR:

Activation of ATM

In response to DNA damage, particularly DSBs, ATM is activated through a series of events:

Detection of DNA Breaks: ATM can sense DNA breaks by associating with the MRN complex (MRE11, RAD50, and NBS1). This complex recognizes DSBs, leading to ATM's recruitment to the damaged site.

Autophosphorylation: Upon detection of DNA damage, ATM autophosphorylates at serine 1981 (S1981), thereby activating it. This phosphorylation event is critical for ATM's kinase activity and its ability to phosphorylate downstream targets.

Dimer-to-Monomer Transition: In its inactive form, ATM is a dimer. It transitions to an active monomeric state upon activation, allowing it to interact with and phosphorylate various downstream proteins.

Phosphorylation Targets of ATM

Once activated, ATM phosphorylates many proteins involved in the DNA damage response, cell cycle regulation, and apoptosis. Here are some critical targets of ATM:

CHK2: ATM phosphorylates CHK2, a cell cycle checkpoint control kinase. This phosphorylation helps to halt the cell cycle, allowing time for DNA repair.

p53: ATM phosphorylates p53, activating this tumor suppressor, leading to cell cycle arrest or apoptosis if the damage is irreparable.

H2AX: ATM phosphorylates histone H2AX, forming γ-H2AX, a marker of DNA damage. This modification helps signal the presence of DSBs and recruit DNA repair machinery.

BRCA1: ATM's phosphorylation of BRCA1 is critical for homologous recombination, a key DNA repair pathway for DSBs.

NBS1: As part of the MRN complex, NBS1 phosphorylation by ATM enhances the complex's DNA repair functions.

53BP1: ATM phosphorylation of 53BP1 helps recruit this protein to sites of DSBs, thereby guiding repair pathways such as non-homologous end joining (NHEJ).

Role of ATM Phosphorylation in DNA Repair

ATM-mediated phosphorylation plays a central role in coordinating the DNA damage response:

Signal Propagation: Phosphorylation cascades initiated by ATM amplify the signal in response to DNA damage, ensuring an efficient response.

Recruitment of Repair Machinery: Phosphorylation of specific proteins helps recruit DNA repair complexes to the sites of DNA damage.

Cell Cycle Regulation: ATM activation can lead to cell cycle checkpoints, preventing cells with damaged DNA from dividing.

Apoptosis: If the DNA damage is too severe to repair, ATM's activation can lead to apoptosis, preventing the propagation of damaged cells

ATM's phosphorylation in response to DNA damage is crucial for detecting DNA double-strand breaks and coordinating the DNA damage response. By phosphorylating a range of critical proteins, ATM orchestrates a complex signaling network that facilitates DNA repair, cell cycle regulation, and apoptosis, thereby maintaining genomic stability.

Method

HeLa cells were grown on acid-etched #1.5H cover glass to ~50% confluency, DNA damage was induced by treatment with 2 μ M Etoposide-spiked complete growth media for 12 hours, followed by -20 °C methanol fixation and blocking with [Thermo Fisher Fish Serum Blocker \(cat#37527X3\)](#). The primary antibody, Rabbit anti-Human ATM.p (Serine 1981) Recombinant Monoclonal, IgG - (BLR031P), was incubated at room temperature for 1 hour at a 1:250 dilution. Cells were washed 3X in PBS. Identifyn® SRM Alexa Fluor™ 647 Affinity Purified Goat Anti-Rabbit, F(ab')₂ (H + L) was incubated at room temperature for 1 hour at a dilution of 1:1000. Cells were washed 5X in PBS.

The second primary antibody, Mouse anti-Human H2Ax Monoclonal, IgG1 - (1H1D9/B7), was then incubated for 1 hour at room temperature at a dilution of 1:250. Cells were washed 3X in PBS.

Identifyn® SRM Alexa Fluor™ 488 Affinity Purified Donkey Anti-Mouse, F(ab')₂ (H + L) was incubated for 1 hour at room temperature at a dilution of 1:1000. Cells were washed 5X in PBS and mounted using [ProLong™ Diamond Antifade Mountant with DAPI cat#P36962](#).

Microscope

Zeiss Axio Observer.Z1/7 Apotome SIM, using an EC Plan-Neofluar 63x/1.25 Oil M27, Axiocam 807 Mono, and X-Cite Xylis Lamp was used with the following parameters for each dye:

Z Stack - (3D) - Panel A, 3-Dimensional Maximum Projection, and Panel C, Z-Plane 33 of 78 Z-Stack = 78 Slices (7.7 μ m)

Channels = 2

Scaling (Per Pixel) = $0.143\ \mu\text{m} \times 0.143\ \mu\text{m} \times 0.100\ \mu\text{m}$
Image Size (Pixels) = 864×791
Image Size (Scaled) = $123.43\ \mu\text{m} \times 113.00\ \mu\text{m}$
Bit Depth = 14 Bit
Image Center Position = X: 50.87 mm, Y: 48.68 mm
Stage Position = X: 50.87 mm, Y: 48.68 mm
ROI Center Offset = X: $1.14\ \mu\text{m}$, Y: $0.00\ \mu\text{m}$

Z Stack - (3D) - Panel B, ROI, 3-Dimensional orthogonal Cut Plane
Z-Stack = 78 Slices ($7.7\ \mu\text{m}$)

Channels = 2
Scaling (Per Pixel) = $0.143\ \mu\text{m} \times 0.143\ \mu\text{m} \times 0.100\ \mu\text{m}$
Image Size (Pixels) = 324×317
Image Size (Scaled) = $46.29\ \mu\text{m} \times 45.29\ \mu\text{m}$
Bit Depth = 14 Bit
Image Center Position = X: 50.87 mm, Y: 48.68 mm
Stage Position = X: 50.87 mm, Y: 48.68 mm
ROI Center Offset = X: $1.14\ \mu\text{m}$, Y: $0.00\ \mu\text{m}$

647 -

Reflector = 50 Cy 5
Beam Splitter = 660
Filter Excitation Wavelength = 625 - 655
Filter Emission Wavelength = 665 - 715
Contrast Method = Fluorescence
Light Source = X-Cite Xylis Lamp Intensity @ 10.00%
Exposure Time = 100 ms
Excitation Wavelength = 653
Emission Wavelength = 668
Effective NA = 1.25
Imaging Device = AxioCam 807 Mono
Camera Adapter = 1X
Depth of Focus = $1.30\ \mu\text{m}$
Binning Mode = 2,2

488 -

Reflector = 38 HE eGFP
Beam Splitter = 495
Filter Excitation Wavelength = 450 - 490
Filter Emission Wavelength = 500 - 550
Contrast Method = Fluorescence
Light Source = X-Cite Xylis Lamp Intensity @ 25.00%
Exposure Time = 250 ms
Excitation Wavelength = 493
Emission Wavelength = 517
Effective NA = 1.25
Imaging Device = AxioCam 807 Mono
Camera Adapter = 1X
Depth of Focus = $1.00\ \mu\text{m}$
Binning Mode = 2,2

Post Processing - SIM Processing

Apotome SIM Image Processing = Display Mode "Optical Sectioning" - enabled correction selected.

Normalization, Phase Corrector, Fourier Filter, and Deconvolution **were NOT used.** **3D Image Processing**

3D Image Processing - Panel A

3D Maximum Projection = Precise

Appearance = Threshold 13% all channels, Ramp 0% all channels, Maximum 100% all channels

Background = Black

Light = Brightness 100%, Enabled Tone Mapping

Projection = View Angle 35°, Scale Z 100%, Reverse Z-Axis NOT Selected, Stereo Anaglyph NOT Selected, Camera Separation 5% (deselected), Parallax Shift 0% (deselected)

Clipping Image Process - Panel A

An X-Y clip plane is introduced in Panel A to highlight the phospho-ATM and the H2Ax within the nuclear compartment.

X-Y = Activate was selected, textured opaque was chosen, and a white outline of the clipped perimeter was shown; Clip Back was selected, and Clip Front was NOT selected.

Position of Clip = -25%

Clip X = 0°

Clip Z = 0°

3D Image Processing - Panel B

3D Maximum Projection = Precise

Appearance = Threshold 13% all channels, Ramp 0% all channels, Maximum 100% all channels

Background = Black

Light = Brightness 100%, Enabled Tone Mapping

Projection = View Angle 35°, Scale Z 100%, Reverse Z-Axis NOT Selected, Stereo Anaglyph NOT Selected, Camera Separation 5% (deselected), Parallax Shift 0% (deselected)

Clipping Image Process - Panel B

Clipping planes are introduced in Panel B to highlight the phospho-ATM and the H2Ax within the nuclear compartment by adjusting or cutting away portions of the image using a triple-wedge cut, removing parts of the image in the X-Y, X-Z, and Y-Z planes.

X-Y = Activate was selected, textured opaque was chosen, and a white outline of the clipped perimeter was shown; Clip Back was selected, and Clip Front was NOT selected.

Position of Clip = -44%

Clip X = 0°

Clip Z = 0°

X-Z = Activate was selected, textured opaque was chosen, and a green outline of the clipped perimeter was shown; Clip Back was selected, and Clip Front was NOT selected.

Position of Clip = 0%

Clip Y = 30°

Clip Z = 0°

Y-Z = Activate was selected, textured opaque was chosen, and a red outline of the clipped perimeter was shown; Clip Back was selected, and Clip Front was NOT selected.

Position of Clip = 0%

Clip Y = -30°

Clip Z = 0°

Single Plane (2D) - Panel C

Shows a two-dimensional view of the original image acquisition at Z-plane 33 of 78 for the phospho-ATM 647 channel alone. The region of interest (ROI) corresponding to the structural analysis shown in Panel B is indicated, providing an isolated view of phospho-ATM aggregate formation and intranuclear organization following etoposide-induced DNA damage.

Summary

Panel A demonstrates a three-dimensional maximum-intensity projection of phospho-ATM and H2Ax following etoposide-induced DNA damage. Rabbit anti-Human ATM.p (Serine 1981) Recombinant Monoclonal, IgG – (BLR031P), visualized using Identifyn® SRM Alexa Fluor™ 647 affinity-purified Goat Anti-Rabbit F(ab')₂ (H + L), demonstrates prominent intranuclear aggregate formation and focal redistribution consistent with activation of the ATM-mediated DNA damage response pathway. Mouse anti-Human H2Ax Monoclonal, IgG1 – (1H1D9/B7), visualized using Identifyn® SRM Alexa Fluor™ 488 affinity-purified Donkey Anti-Mouse F(ab')₂ (H + L), remains broadly distributed throughout the chromatin compartment, reflecting the constitutive nuclear localization of H2Ax as a histone substrate involved in DNA damage signaling. An X-Y clip plane positioned at -25% improves visualization of the internal nuclear organization, while the boxed region identifies the area selected for further volumetric analysis in Panel B.

Panel B presents a three-dimensional wedge-view orthogonal reconstruction of the selected region of interest. Orthogonal projections across the X-Y, X-Z, and Y-Z planes demonstrate strong spatial proximity between phospho-ATM and H2Ax within DNA damage-associated nuclear territories. Biologically, ATM phosphorylation is rapidly induced following DNA double-strand break formation and functions as a central upstream regulator of checkpoint activation, chromatin signaling, and DNA repair coordination. ATM-mediated phosphorylation of H2Ax into γH2AX represents one of the earliest amplification events in DNA damage signaling; however, because the present dataset measures total H2Ax rather than γH2AX specifically, the observed overlap likely reflects broader chromatin-associated recruitment and DNA damage-associated nuclear organization rather than direct molecular colocalization. The orthogonal projections confirm that the observed signal overlap is present throughout the three-dimensional nuclear volume and not solely due to the planar projection artifact.

Panel C provides a two-dimensional view of the original acquisition at Z-plane 33 of 78 for the phospho-ATM 647 channel alone, with the region of interest corresponding to Panel B identified. This isolated phospho-ATM view demonstrates extensive aggregate and punctate signal formation following etoposide treatment, consistent with localized activation and concentration of ATM signaling domains at sites of DNA damage response activity.

Collectively, the dataset demonstrates robust nuclear recruitment and redistribution of phospho-ATM following genotoxic stress, accompanied by strong apparent overlap with

H2Ax-containing chromatin territories. At diffraction-limited and near-diffraction-limited resolution, these modalities are highly effective for assessing global response patterns, aggregate formation, and spatial nuclear organization. However, because total H2Ax is broadly distributed throughout chromatin and because widefield and volumetric reconstructions remain structurally conflated at the nanoscale, the observed overlap should be interpreted as coordinated engagement within shared DNA damage response microenvironments rather than definitive molecular interaction. Higher-resolution super-resolution approaches remain necessary to resolve the underlying nanoscale architecture and true structural relationships between ATM signaling complexes and phosphorylated chromatin domains.

Image Correction

NONE

Image by E.F.Simon

Image Analysis and Data Presentation by B.T. Bennett

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