

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 signals the presence of DSBs and recruits 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 Imager.M1 Widefield, using a Plan-Apochromat 63x/1.40 Oil M27, Axiocam 705, and X-Cite Xylis Lamp, was used with the following parameters for each dye:

Single Plane (2D)

Channels = 3

Scaling (Per Pixel) = 0.110 μ m X 0.110 μ m

Image size (Pixels) = 680 X 559

Image Size (Scaled) = 74.48 μm X 61.22 μm
Bit Depth = 14 Bit
Image Center Position = X: 0.00 mm, Y: 0.00 mm
ROI Center Offset = X: 0.00 μm , Y: 0.00 μm

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 @ 15.00%
Exposure Time = 150 ms
Excitation Wavelength = 653
Emission Wavelength = 668
Effective NA = 1.4
Imaging Device = AxioCam 705
Camera Adapter = 1X
Depth of Focus = 1.03 μm
Binning Mode = 2,2

488 -

Reflector = 38 eGFP
Beam Splitter = 495
Filter Excitation Wavelength = 450 - 490
Filter Emission Wavelength = 500 - 550
Contrast Method = Fluorescence
Light Source = X-Cite Xylis Lamp Intensity @ 15.00%
Exposure Time = 150 ms
Excitation Wavelength = 493
Emission Wavelength = 517
Effective NA = 1.4
Imaging Device = AxioCam 705
Camera Adapter = 1X
Depth of Focus = 0.80 μm
Binning Mode = 2,2

DAPI -

Reflector = 49 DAPI
Beam Splitter = 395
Filter Excitation Wavelength = 335 - 383
Filter Emission Wavelength = 420 - 470
Contrast Method = Fluorescence
Light Source = X-Cite Xylis Lamp Intensity @ 10.00%
Exposure Time = 30 ms
Excitation Wavelength = 353
Emission Wavelength = 465
Effective NA = 1.4
Imaging Device = AxioCam 705
Camera Adapter = 1X
Depth of Focus = 0.72 μm
Binning Mode = 2,2

Split View - Panel A

The top-left panel shows 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). The top-right panel shows 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). The lower-left panel shows DAPI nuclear staining, and the bottom-right panel shows the three-channel merged image.

Colocalization View - Panel B

Horizontal Axis - AF647, Threshold 190, Range Auto - Phospho-ATM
Vertical Axis - AF488, Threshold 332, Range Auto - H2Ax
Image Measurement = Entire Field

Colocalization analysis of Phospho-ATM (X-axis) and H2AX (Y-axis) was performed across the entire image acquisition.

Summary

Panel A shows a split-view widefield acquisition following etoposide-induced DNA damage. The upper left panel shows Rabbit anti-Human ATM.p (Serine 1981) Recombinant Monoclonal, IgG – (BLR031P), detected using Identifyn® SRM Alexa Fluor™ 647 Affinity Purified Goat Anti-Rabbit F(ab')₂ (H + L). The upper right panel shows Mouse anti-Human H2Ax Monoclonal, IgG1 – (1H1D9/B7), detected using Identifyn® SRM Alexa Fluor™ 488 Affinity Purified Donkey Anti-Mouse F(ab')₂ (H + L). The lower-left panel shows DAPI nuclear staining, while the lower-right panel shows the three-channel merged image. Following etoposide exposure, phospho-ATM demonstrates strong intranuclear aggregate formation and large punctate foci consistent with activation of the DNA damage response. H2Ax signal remains broadly distributed throughout the nucleus with overlapping enrichment in damage-associated regions.

Panel B presents colocalization analysis of the same acquisition, with phospho-ATM plotted along the horizontal (X) axis and H2Ax plotted along the vertical (Y) axis. Strong correlated localization is observed between both signals, consistent with coordinated recruitment within DNA damage-associated nuclear domains following genotoxic stress. Biologically, ATM phosphorylation is rapidly induced at DNA double-strand breaks and functions upstream in checkpoint activation and DNA damage signaling. H2Ax serves as the substrate pool from which γH2AX is generated following ATM- and ATR-mediated phosphorylation at sites of DNA damage. As such, broad spatial overlap between phospho-ATM and total H2Ax is expected at widefield resolution due to the dense nuclear distribution of H2Ax chromatin and the large-scale organization of damage-associated signaling regions.

Importantly, while widefield microscopy demonstrates strong apparent colocalization and provides valuable information regarding protein response, nuclear localization, and aggregate formation, the modality remains diffraction-limited and unable to resolve nanoscale structural relationships. As resolution increases through super-resolution modalities, apparent colocalization observed at widefield scales becomes progressively resolved into structurally distinct subdomains. This distinction is biologically important, as phospho-ATM signaling is more directly associated with γH2AX-enriched damage

regions rather than total H2Ax distribution alone. Therefore, the strong colocalization observed here should be interpreted as coordinated engagement within shared DNA-damage response territories rather than as definitive molecular overlap. Widefield imaging remains highly effective for assessing global response patterns, aggregate formation, and intranuclear redistribution. At the same time, higher-resolution modalities are required to resolve the underlying nanoscale architecture of these signaling complexes.

Image Correction

NONE

Image by E.F.Simon

Image Analysis and Data Presentation by B.T. Bennett

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