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Determining the Role of ATM in Claspin Activation and Localization

Author
Gladys, Devin Latrell

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Determining the Role of ATM in Claspin Activation and Localization

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by

Devin Latrell Gladys

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ABSTRACT OF THE THESIS

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Devin Latrell Gladys

Master of Science in Molecular Biology

University of California, Los Angeles, 2019

Professor Feng Guo, Chair

Claspin is a Chk1 regulatory protein that acts at replication forks in both the DDR and replication pathways to ensure the fidelity of DNA prior to entry into mitosis and to initiate DDR in the case of damaged DNA. Though many of Claspin's functional domains have been identified it is unclear what activates and localizes it to DNA. ATM is a kinase that regulates the replication and DDR pathways at SQ and TQ sites. Here we propose using caffeine, an inhibitor of ATM, to determine the importance of ATM in Claspin activation and localization, and following that with a combination of rescue experiments with Claspin deficient mice that utilizes phospomimetic and unphosphorylatable versions of Claspin to determine the importance of phosphorylation at ATM targets sites for Claspin activation. We will then use immunocytochemistry to determine if Claspin mutants are successfully recruited to DNA.

The thesis of Devin Latrell Gladys is approved.

Peter Bradley

Jeffrey Long

Feng Guo, Committee Chair

University of California, Los Angeles

2019

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Specific Aims:

Claspin is a Chk1 regulatory protein that is highly conserved in eukaryotes and uses a ring-like structure to bind to chromatin near the replication origin. Chk1 inhibits entry into mitosis when replication requirements are not met; preventing the progression of mitosis is important in the event of DNA damage, which makes studying Claspin worthwhile because of its necessity in ordinary division of cells and the impact it might have on cancer and disease related research. Ataxia telangiectasia and Rad3-related protein (ATR) are necessary for phosphorylation and activation of Chk1, and it is thought that Claspin works in concert with ATR and Rad17 to sense unique aspects of the DNA replication fork. However, it has been suggested that ATR is not necessary for Claspin to bind to at replication forks. (Ataxia-telangiectasia mutated) ATM is a protein kinase that is a central mediator of the DNA damage response and phosphorylates various proteins following ionizing radiation damage. Since ATM and ATR are both PIK kinases and function in the same pathway, it raises the question of whether phosphorylation by ATM is necessary for Claspin activation and localization to DNA. While many binding domains and post-translational modification sites in Claspin have been identified, it is unclear whether ATM phosphorylates any sites on Claspin. Determining what localizes Claspin to DNA would give us a better understanding of its role in the DDR pathway and cell replication in general, since replication forks, where Claspin acts, are necessary in both processes. ATM phosphorylates at serine glutamine (SQ) and threonine glutamine (TQ) sites, and Claspin has both SQ and TQ sites that are phosphorylated. We hypothesize that phosphorylation by ATM is necessary to activate Claspin to allow it to bind DNA. To test this, we will employ genetic mice models, novel Claspin mutants, mass spectrometry, and fluorescence microscopy.

Specific Aim 1: Identify phosphorylation sites on Claspin and determine whether phosphorylation of any of these sites is inhibited by caffeine, an inhibitor of ATM. Claspin has several SQ and TQ sites that could serve as ATM phosphorylation sites. To determine if

any of these sites are in fact phosphorylated upon replication, we will put H293T cells under oxidative to activate ATM and induce DNA damage, and then isolate Claspin from these cells. Using tandem mass spectrometry (MS/MS) sequencing we will identify the phosphorylation sites in Claspin. Following that, we will repeat the same experiment in the presence of caffeine, a known inhibitor of ATM/ATR, to determine if phosphorylation of any of these sites is inhibited by treatment with caffeine. Since caffeine is an inhibitor of both ATM and ATR these experiments will be carried out in cells where ATR has been silenced in order to ensure that sights left un-phosphorylated are indeed due to ATM inhibition. Expected outcome: we expect that ATM target sites on Claspin will remain un-phosphorylated in the presence of caffeine indicating that ATM does in fact phosphorylate Claspin.

Specific Aim 2: Assess whether Claspin mutants can rescue Claspin deficient flox mice.

Once we have identified the ATM phosphorylation sites on Claspin we will generate mutants that cannot be phosphorylated. These Claspin analogues should have reduced Chk1 activity, resulting in a phenotype that cannot recover from DNA damage or progress through mitosis as efficiently as wild type. It has been shown that homozygous Claspin mutants ($Claspin^{-/-}$) are lethal, so in order to create an environment depleted of wild type (wt) Claspin we will use mouse embryonic fibroblasts (MEF) from previously engineered Claspin flox mice. In these mice adenovirus encoding Cre recombinase (Ad-Cre) can be used to knockout wt Claspin after the mice have reached appropriate maturity. Then we would transfect mutant Claspin into MEFs and induce oxidative stress to determine if the mutants can rescue the MEFs. We would repeat these experiments with a phosphomimetic mutant of Claspin as well. Expected outcome: we expect that the wt MEFs will be unable to recover from DNA damage and we expect the un-phophorylatable Claspin mutant to share that phenotype. In contrast, we expect the phosphomimetic Claspin mutant to be able to recover from DNA damage because it is essentially a constitutively active mutant.

<u>Specific Aim 3:</u> Use immunocytochemistry to determine if the Claspin mutants are successfully recruited to DNA. To track Claspin's movement, we will use antibodies with a fluorescent moiety to pinpoint the subcellular location of Claspin. In this experiment, we will use Ad-Cre to deplete wt Claspin in MEFs, transfect the appropriate mutant, induce DNA damage, and then visualize the damaged cells using fluorescence microscopy. This will allow us to determine if Claspin that cannot be phosphorylated by ATM still localizes to DNA at sites of replication. Expected outcome: the phosphomimetic Claspin will localize to DNA normally, although likely in greater abundance since it will be constitutively active. We expect the unphosphorylatable Claspin to be degraded or to localize elsewhere. If both Claspin mutants still localize to DNA then it is possible that ATM may be involved in activation of Claspin but not in identification of/localization to damaged and replicating DNA.

Significance

The DNA replication pathway is a critical step in the propagation of cells through cell division. However, normal cells show arrested development when their DNA is damaged[1]. Therefore, the DDR pathway is essential for dividing cells and cells where DNA damage occurs. Damaged DNA can occur from several different sources such as oxidative stress, ultra-violet radiation, and hydrolytic damage[1]. The DDR is able to handle all of these different types of DNA damage thanks to the myriad of repair proteins in this pathway. These proteins are responsible for recognizing, unwinding, annealing to, and repairing damaged DNA. Both the DDR and replication pathways are highly conserved, and some proteins in these pathways have been identified as oncogenic[2]. There is clear interest in these pathways and the proteins involved because of the insights they may give us into cancer and possible therapeutics.

ATM is a member of the family of phosphoinositide-3-kinase-related kinases (PIKKs) that regulates both cell cycle progression and DNA repair[3,4]. It is at the top of a signaling cascade and modulates many proteins that are involved in both of these pathways[5]. Chk1 is a

checkpoint protein that regulates the progression of the cell cycle and inhibits initiation of mitosis when there is incompletely replicated DNA present[6]. Claspin is an essential activator of Chk1 that binds DNA in a ring-like structure[7]. Many of Claspin's functional domains, such as the Chk1 binding domain, have been identified, but it is still unclear what activates it or localizes it to DNA[8]. Both ATM and ATR, another PIKK, are kinases involved in pathways with Claspin, however, it has been shown that Claspin can function independently of ATR in yeast[9,10]. While it has been shown that Claspin interacts with ATR, there has been no direct evidence of a physical interaction between Claspin and ATM[11]. The ATM/ATR kinases phosphorylate at serines and threonines that are followed by glutamines[3]. There are several SQ and TQ sites in Claspin that could serve as potential targets of ATM, and it has been shown that some of them are in fact phosphorylated[12]. Research has shown that phosphorylation can impact protein function, localization, and binding, and phosphomimetics and unphosphorylatable versions of proteins have been shown to have differential function when compared to wild type[13,14]. Since ATM is the primary signaler in both the replication and DDR pathways, we hypothesize that it is responsible for activating Claspin and localizing it to damaged DNA.

Determining what activates Claspin is important because it regulates a "checkpoint" protein and is involved in indispensable biological processes. We plan to identify phosphorylation sites in Claspin and determine whether these sites are targets of ATM, and whether phosphorylation by ATM is required for Claspin to interact with chromatin. Previous research has shown that Claspin is an essential regulator of cell cycle progression and is an integral component of the DDR pathway. Research into Claspin's mechanism of action may provide new insights in dealing with cancer growth and viral infections considering their reliance on these pathways.

Innovation

In Aim 1 we intend to identify phosphorylation sites in Claspin and determine which of these sites are potentially phosphorylated by ATM. The ATM pathway can be activated by inducing oxidative stress using H_2O_2, and oxidative stress can also induce DNA damage which requires recruitment of Claspin for repairs[15,16]. We will expose normal human dermal fibroblast cells to oxidative stress and use Claspin specific antibodies to purify human Claspin[17]. ATM and ATR are both involved in DNA damage and share some substrates, however, ATR is primarily active at stalled replication forks[1]. In order to ensure that the phosphorylation we see is due to ATM and not ATR, we will silence ATR in these cells using previously developed siRNA[18]. We will use tandem mass spectrometry to isolate and identify phosphorylated regions of human Claspin. Next, we will repeat these experiments in the presence of caffeine, an ATM/ATR inhibitor, and exam any differences in phosphorylation patterns[9]. In the samples exposed to caffeine we expect to find TQ and SQ sites have not been phosphorylated because of ATM inhibition. This unique experimental approach exploits the Claspin and ATM pathways to determine specifically which sites on Claspin ATM might target, while also eliminating ATR kinase activity as a variable.

Aim 2 assesses whether Claspin with mutated target TQ and SQ sites, which will have been identified by Aim 1, can rescue wt Claspin depleted mice embryonic fibroblast cells (MEFs). Using a Claspin Cre-loxp system developed by Yang *et al*. we will deplete wt Claspin in MEFs prior to inducing oxidative stress[17]. We will infect cells with Ad-Cre to knockout wild type Claspin. We will then transfect the unphosphorylatable mutant Claspin, which will be generated using sited directed mutagenesis to mutate target T or S sites to alanines. As a positive control, we will generate phosphomimetic analogues of our mutant Claspin, by either mutating from threonine to aspartic acid or from serine to aspartic acid. These phosphomimetic mutants should be constitutively active and therefore be able to rescue the wt Claspin depleted MEFs.

These novel mutants will allow us to assess whether human Claspin mutants can rescue the MEFs and essentially answer whether ATM is necessary for the activation of Claspin.

In Aim 3 we wish to elucidate whether the Claspin mutants are able to localize to DNA. We will use immunocytochemistry to track the location of our novel phosphomimetic mutants and their unphosphorylatable counterparts. We will infect MEFs with Ad-Cre to deplete wt Claspin, transfect the appropriate mutant, induce oxidative stress and then determine whether the Claspin mutant successfully localizes to DNA. As a negative control, we will repeat this experiment with un-transfected MEFs. We will incubate the cells with caffeine and visualize them to determine whether Claspin can localize to chromatin. In order to be consistent with other experiments, ATR will be silenced in all of these experiments. This unique approach allows us to assess without a doubt whether phosphorylation by ATM is essential for Claspin localization at replication forks. Use of these novel mutants will allow us to narrow our focus to ATM without being concerned about whether ATR is also having an effect at these sites. Overall, this innovative experimental procedure seeks to identify ATM target sites on Claspin and establish whether ATM is indispensable for Claspin activation, while also accounting for the fact that ATM and ATR have similar substrates and target the same motifs.

Experimental Design

Specific Aim 1: Identify phosphorylation sites in Claspin and determine whether phosphorylation of any of these sites is inhibited by caffeine, an inhibitor of ATM.

Rationale and Hypothesis. ATM is a PIK kinase that regulates the DDR response and is known to activate many proteins necessary for the DDR and regular progression through the cell cycle[19,20]. Claspin interacts with ATR in order to regulate Chk1, which is also involved in ensuring the integrity of DNA before replication[21]. The activator of Claspin has not been identified, although it is possible that ATM is responsible for its activation. Claspin operates in the same pathways that ATM regulates, and although there has been no direct evidence of

physical interaction between the two, Claspin is phosphorylated upon activation and there are several potential ATM target sites on Claspin[12]. Therefore, we propose that ATM is responsible for some of the phosphorylation seen in Claspin. ATM targets serine glutamine and threonine glutamine sites, so assessing phosphorylation at these sites in the presence and absence of an ATM inhibitor will allow us to determine whether ATM is responsible for modifying any of these SQ and TQ sites.

Objective 1.1. Expression and purification of human Claspin from H293T cells. We will follow a previously published protocol by Uno et al. on how to efficiently express and purify Claspin and other large size proteins in mammalian cells[22]. Briefly, a CSII-EF-MCS-mAG-6His-Claspin-3Flag plasmid containing full length human Claspin obtained from Dr. Syuzi Uno will be transfected into H293T cells using polyethylenimine "MAX" reagent (PEI) (Figure 1A). 0.8 ug of plasmid DNA will be dissolved in 50 uL of 150 mM NaCl (solution "A") and 4 uL of 1 mg/mL of PEI solution will be mixed with 50 uL of 150 mM NaCl (solution "B"). These two solutions will be gently mixed and incubated at room temperature for 30 min. After incubation the solution will be added to a twelve well plate containing H293T cells grown in 1 mL of fresh D-MEM. mAG fluorescence will be assessed at 488 nm 42 h after transfection and incubation at 37 ˚C in 5% CO_2. For Claspin extraction and purification cells will be harvested from a 15 cm plate 42 h after transfection using the previously described protocol[22]. Claspin will then be purified using a Ni affinity column, TEV protease will be used to cleave the mAG site, and Claspin will be further purified using anti-FLAG M2 affinity beads. Eluted Claspin fractions will be analyzed using SDS-PAGE and Coomassie Blue staining.

Expected Results, Interpretation, and Possible Pitfalls. Successful transfection of the Claspin plasmid into the H293T cells will be assessed by determining mAG fluorescence under microscope when excited at 488 nm. Transfection efficiency will be calculated as a percentage of cells displaying mAG fluorescence as compared to the total population of cells. Transfection efficiency will be quantitatively analyzed by fluorescence activated cell sorting (FACS). Although

it is unlikely because this protocol has already been optimized for the efficient transfection of the Claspin plasmid in mammalian cells, a low transfection yield is one possible pitfall of the experiment. In this case, we would adjust the transfection protocol (such as transfection reagent, plasmid, incubation time and temperature) until we achieve a transfection efficiency of at least 50%.

Objective 1.2. Assessing differential Claspin phosphorylation by ATM in the presence of caffeine by tandem mass spectrometry. To determine what level of Claspin phosphorylation is due to ATM, cells were co-transfected with ATR-1 siRNA (Table 1) and Claspin, and then transfected with ATR-2 siRNA within 24 h[22,23]. Cells were grown for 42 h, then treated with 200 mM caffeine 30 min prior to treatment with H_2O_2[24]. To induce oxidative stress cells were incubated in media containing 25 uM H_2O_2 for 1 h[16]. Next, cells were prepared for MS/MS sequencing as previously described by Breitkopf *et al*[25]. One population of cells will be treated with caffeine and H_2O_2, while one set of cells will be treated with H_2O_2. Briefly, Claspin will be purified from both the caffeine treated and untreated cells as described above, separated on an SDS gel, excised from said gel, submitted for MS/MS analysis, and then compared for differential phosphorylation using the program Sequest.

Expected Results, Interpretation, and Possible Pitfalls. We expect that some phosphorylation sites on Claspin will be left unphosphorylated in the caffeine treated samples. To ensure that ATR is being silenced its protein expression will be measured using western blotting and anti-ATR antibodies. Once ATR silencing is confirmed samples will be analyzed using MS/MS and comparing the phosphorylation in caffeine treated and control samples. This will allow us to identify sites whose phosphorylation depends on ATM. A possible pitfall of this experiment is that we will not observe any differential phosphorylation between the two sample populations. In that case, we will conduct an experiment where we silence ATM and look at phosphorylation between an ATM depleted sample, and a control sample only treated with H_2O_2. If the two samples are identical then it is unlikely that ATM is responsible for phosphorylating sites on

Claspin; however, if the amount of phosphorylation differs between the two samples then it is likely that ATM is involved in Claspin phosphorylation and we will adjust our conditions to optimize ATM inhibition until we see differential phosphorylation in caffeine treated samples. We may also consider other ATM inhibitors to see if they give us better inhibition.

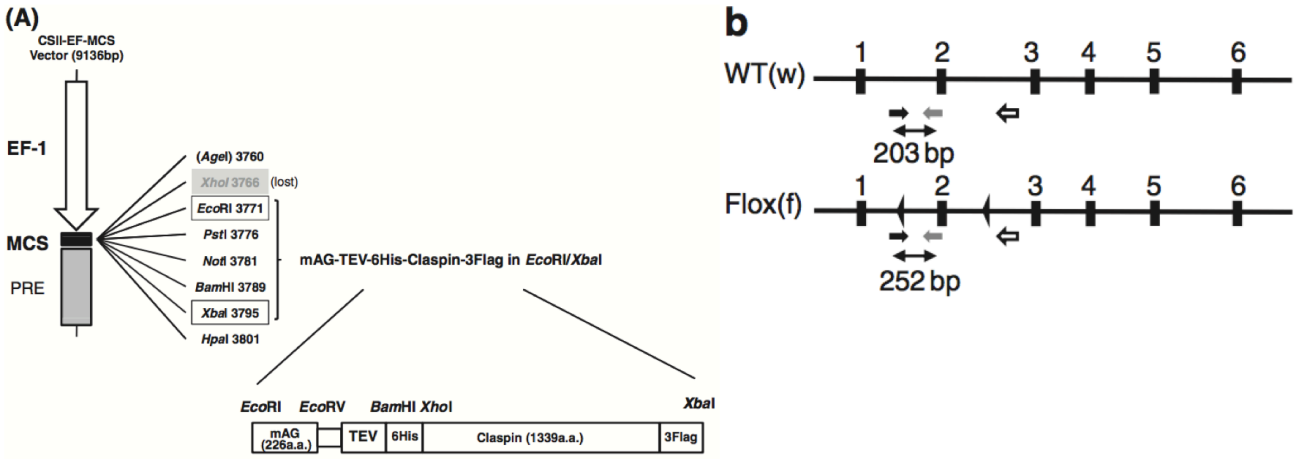

Figure 1. Claspin Plasmid construction and flox knockout mouse schematics. A) Schematic depicting CSII-EF-MCS vector. Claspin is flanked by a his- and Flag-tags. A TEV protease site follows the mAG sequence for easy removal. B) Diagram of WT and Flox Claspin genes. Exon two is removed upon infection with Ad-Cre. Arrows represent primers used for genotyping. Adapted from Uno *et al*. and *Yang et al*[17,22].

Specific Aim 2: Assess whether Claspin mutants can rescue Claspin deficient flox mice.

Rationale and Hypothesis. Claspin is fairly conserved in mammalian cells and based on BLAST alignments human and mouse Claspin share a 74% sequence similarity; this degree of similarity suggests that human Claspin might be able to rescue Claspin deficient mouse embryonic fibroblasts. If human Claspin can rescue Claspin$^{flox/-}$ MEFs then we can use this system to study Claspin mutants with modified ATM target sites. For example, we can examine the effect phosphomimetic and unphosphorylatable Claspin analogues affect the growth of Claspin$^{f/-}$ MEFs and whether the Claspin analogues are still localized to DNA. The Claspin analogue with phosphomimetic ATM target sites, which would be identified using mass spec, would be expected to be constitutively active and be able carry out normal Claspin function.

Claspin engineered to be unphosphorylatable at ATM target sites is predicted to be inactive. Here, we hypothesize that human Claspin can rescue Claspin deficient MEFs. We also hypothesize that phosphomimetic Claspin will be able to rescue MEFs, while unphosphorylatable Claspin will not be able to.

Objective 2.1: Determine if human Claspin can rescue Claspin deficient MEFs. A Claspin flox/- mice line was developed by Yang *et al* and MEFs bearing this genotype will be obtained from this group (Figure 1B)[23]. We will genotype the MEFs using the protocol outlined by Yang *et al*. Briefly, MEFs will be lysed in 500 uL of lysis buffer (50 mM Tris–HCl (pH 8.0), 100 mM NaCl, and 20 mM EDTA) with 500 uL/mg Proteinase K for 20 h at 55 ˚C. Next, a phenol/chloroform extraction (1:1) will be performed and DNA will be recovered using ethanol extraction. The final pellet to be used for PCR will be dissolved in 100 uL of tris-EDTA buffer[23]. Once the genotype has been confirmed, we will use the previously described protocol to transfect the CSII-EF-MCS-mAG-6His-Claspin-3Flag plasmid into MEFs[22]. Ad-cre will then be administered to normal flox MEFS cells transfected with the Claspin plasmid.

Expected Results, Interpretation, and Possible Pitfalls. We expect that the Claspin$^{f/-}$ MEFs will experience impaired growth and DNA synthesis, whereas the MEFs transfected with the Claspin plasmid will be will still be able to grow normally. FACS will be used to assess successful incorporation of the Claspin plasmid, while a BrdU assay will be used to compare growth and DNA synthesis of the untreated flox MEFs, Ad-Cre infected MEFs$^{f/-}$, and the MEFs$^{f/-}$ transfected with the Claspin plasmid. The uninfected MEFs$^{f/-}$ will serve as a positive control, and the Ad-Cre infected MEFs$^{f/-}$ will be a negative control. This will allow us to assess cell growth in our transfected MEFs as compared to cells completely lacking Claspin and those with a wt Claspin phenotype. Cell proliferation will be assessed using a BrdU assay and FACS which measures cell proliferation via DNA synthesis. Proliferating cells are synthesizing new DNA and will incorporate the thymidine derivative BrdU, which can then be imaged using a fluorescent monoclonal BrdU antibody[22,26]. We expect uninfected MEFs$^{f/-}$ to display the most fluorescence,

the Ad-Cre infected MEFs$^{f/-}$ to show little to no fluorescence, and the Claspin transfected, Ad-Cre infected MEFs$^{f/-}$ to display an amount of fluorescence somewhere between the two. A major possible pitfall for this experiment is that human Claspin may not be able to rescue the MEFs. If this is the case, we will perform the same experiments in H293T cells where we use siRNA to silence Claspin. While this approach should produce the same functional data, performing these experiments in MEFs would allow us to prove whether human Claspin can perform the function of mouse Claspin and give us a clean system where we know that any endogenously produced Claspin is likely nonfunctional.

Objective 2.2: Generate unphosphorylatable and phosphomimetic Claspin analogues. After determining ATM target sites on Claspin, we will use primer extension PCR site directed mutagenesis to introduce substitutions at those sites in our Claspin vector[27,28,29]. The ATM target sites will be mutated to either aspartic acid (phosphomimetic Claspin) or alanine (unphosphorylatable Claspin). The CSII-EF-MCS-mAG-6His-Claspin-3Flag plasmid will be digested with XhoI and XbaI to isolate the flag-tagged Claspin sequence. Two sets of primers will be used in these PCR reactions; primers “A” and “D” (Table 1) will encompass the entire Claspin sequence and primers “B” and “C” will target overlapping regions and contain a mutation in their sequences. The first round of PCR will generate partial products with the desired mutation and the second round of PCR will be done with just the “A” and “D” primers to generate the full-length Claspin with desired mutations. This process will be repeated until all desired mutations have been incorporated into Claspin. Only one mutation will be introduced at a time to keep primer sizes relatively small and reduce the chance of poor annealing efficiency. Final gene products will be cloned in-frame back into the expression vector at the XhoI and XbaI sites (Figure 1A).

Expected Results, Interpretation, and Possible Pitfalls. Following generation of the Claspin mutants they will be sent off for sequencing to confirm that they are the constructs we intended to create. Next, the transfection efficiency of the new vectors will be assessed by looking at

mAG fluorescence in cells, and the protein expression will be determined using western blotting. A possible pitfall of this experiment is that the protein expressed by the newly generated Claspin vectors will not be stable. If that is the case, we will determine the maximum number of mutations that still produces stable Claspin by mutating one site at a time and testing its expression and stability using western blotting and a gel mobility shift assay.

Objective 2.3: Determine if Claspin mutants can rescue Claspin$^{f/-}$ MEFs. To evaluate whether the phospomimetic and unphosphorylatable mutants can rescue the Claspin$^{f/-}$ MEFs we will utilize the same procedure used to determine if human Claspin can rescue MEFs. The plasmids will be transfected into MEFs using PEI transfection reagent and successful transfection will be determined by analyzing mAG fluorescence at 488 nm[22]. Successful transformants will be re-plated, infected with Ad-Cre, and incubated with H_2O_2 to stimulate oxidative stress[16]. From there, cell growth and DNA synthesis will be evaluated using a BrdU assay[26].

Expected Results, Interpretation, and Possible Pitfalls. We expect that the cells transfected with the phosphomimetic Claspin will be constitutively active and be able to perform DNA damage repair and progress through the cell cycle. In contrast, we expect that the MEFs transfected with unphosphorylatable Claspin will have impaired growth and DNA synthesis. Untransfected MEFs$^{f/-}$ not treated with Ad-Cre will serve as a positive control, while untransfected, Ad-Cre MEFs will serve as a negative control. Growth/DNA synthesis will be assessed using a BrdU assay. If the steps leading up to this experiment are successful, we expect to have addressed any pitfalls that may occur because of impaired growth or Claspin expression. If the levels of growth in the two mutants are similar we will scale of the number of cells analyzed and see if any nuanced differences can be detected.

Specific Aim 3: Use immunocytochemistry to determine if the Claspin mutants are successfully recruited to DNA. ***Rationale and Hypothesis***. We hypothesize that ATM phosphorylation is not only necessary for the activation but also the localization of Claspin. Our

initial experiments are focused on demonstrating whether ATM is required for the normal function of Claspin, but here we will focus on determining the subcellular location of Claspin with and without ATM inhibition. If phosphorylation of Claspin by ATM is necessary for its activation then it is possible that some of the modifications ATM produces in Claspin are involved in its ability to bind to DNA. We will use immunocytochemistry to visualize the subcellular location of our Claspin mutants after inducing oxidative stress in the cells.

Objective 3: Visualize subcellular localization of Claspin mutants using immunocytochemistry.

To determine the subcellular location of our Claspin mutants we will transfect the plasmids containing out phospomimetic and unphosphorylatable mutants into H293T cells. Following transfection cells will be seeded onto coverslips sterilized in 70% ethanol, grown to confluency, infected with Ad-Cre, and incubated with H_2O_2. Cells will then be washed in PBS, incubated in ice-cold 5% methanol for 5 min, washed in PBS, and blocked for 1 h in 10% FCS with 1 mg/mL BSA in PBTx. Cells will then be incubated for 1 h with Claspin antibody followed by a 1 h incubation in the Alexa Fluor 568 goat anti-human secondary antibody[30,31]. Cells will then be analyzed under fluorescent microscope to determine the location of Claspin within the cell. Controls will include un-transfected MEFs, cells treated with only secondary antibody, cells treated with only primary antibody, and cells not treated with any antibody[32].

Expected Results, Interpretation, and Possible Pitfalls. We will interpret our results by comparing the physical location within in the cells of both Claspin mutants. We expect the phosomimetic Claspin to be localized to the nucleus where DNA is stored. We expect the unphosphorylatable Claspin mutant to be found elsewhere in the cell, since it would be unable to localize to DNA because it cannot be activated by ATM. The un-transfected MEFs will provide us with an example of how much Claspin is normally associated with DNA following oxidative stress and ATM activation. The other controls will test specificity of the antibodies and background signal to be expected. Densitometric fluorescence analysis will be performed on the images to quantify the amount of Claspin signal at each location. One possible pitfall is that we

may see Claspin signal from the unphosphorylatable mutant at the nucleus. If this is the case, it is possible that phosphorylation by ATM is not responsible, or soley responsible, for localizing Claspin to the nucleus.

Table 1. Sequences of relevant oligonucleotides*

Name	Sequence (5'-3')
Claspin Plasmid F-Primer "A"	cg-(XhoI)-atgacaggcgaggtgggttc
Claspin Plasmid R-Primer "D"	cg-(XbaI)-tta-(NheI)-gctctccaaatatttgaag
ATR-1 siRNA	CCUCCGUGAUGUUGCUUGA
ATR-2 siRNA	CCCGCGUUGGCGUGGUUGA

*Non-exhaustive list. Other necessary nucleotides will be determined after relevant Claspin phosphorylation sites are determined. Adapted from Yang *et al*. and Uno *et al*[17,22].

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