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# MacroH2A1 Regulation During the Cell Cycle of Mouse Embryonic Stem Cells

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## APPROVAL PAGE

## HONORS THESIS

MacroH2A1 REGULATION DURING THE CELL CYCLE OF MOUSE EMBRYONIC  
STEM CELLSPresented by  
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***Abstract***

MacroH2A is a core histone variant that plays an important role in the X-inactivation process during differentiation of embryonic stem cells. It has been shown that macroH2A changes in localization during the cell cycle of somatic cells. This study aims to determine how macroH2A changes during the cell cycle of embryonic stem cells. Male and female mouse embryonic stem cells were transfected with a GFP::macroH2A construct and the relationship between macroH2A and the cell cycle was determined using FACS. This study shows that macroH2A is altered during the cell cycle of embryonic stem cells as it is in somatic cells and that in randomly cycling cells, there is a correlation between macroH2A expression and the phases of the cell cycle. High GFP expressing cells are mostly in the G2/M phase and low GFP expressing cells are mostly in the G1 phase. This correlation indicated that macroH2A is replicated with cellular DNA during the S phase resulting in higher expression in the G2/M phase. Future research, such as RT-PCR and differentiation experiments, is needed to further study this relationship and determine whether this change is at the protein or RNA level and how it changes during differentiation.

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## ***Introduction***

Epigenetics is an area of research that focuses on heritable changes in phenotypic traits that are not based on DNA sequence, but instead involve chromatin remodeling controlled by histone protein or histone protein variant modification. Epigenetics is important in many biological processes, especially in the process of development. The body is composed of highly specialized cells that make up different tissues and organs. However, these cells all develop from the totipotent embryonic stem cells of the zygote and contain essentially the same genetic content. What makes them distinct and functional are epigenetic modifications that regulate DNA expression. Thus, epigenetics is an area of particular interest in the field of stem cell research because it provides great insight into understanding development and replicating this process in vitro. Many of the current mechanisms of mammalian reprogramming, including somatic cell nuclear transfer and induced pluripotent stem cells, are believed to involve epigenetic modifications (Ambrosi et al, 2007).

The basis of epigenetics lies at the nucleosome—the organizational unit of chromatin. Nucleosomes are composed of DNA wound around core histone proteins that keep the cell's large quantity of DNA condensed and ordered (Imhof et al, 2006). Two copies each of the four core histone proteins, H2 (H2A and H2B), H3 and H4, form the octamer centers of nucleosomes (Figure 1). H1 is a linker protein in the nucleosome involved with chromatin condensation. Each of these histones contains a core structural motif called the histone fold domain, flanked by N- and C-terminal tails. Epigenetic modifications such as acetylation, methylation, ubiquitination, phosphorylation and ADP-ribosylation occur at the histone tails. Each tail has many modification sites and each

modification can have a different effect on the physical characteristics of the nucleosome (Chadwick et al, 2001).

Numerous variants of the core histone proteins have been identified and have been shown to be able to both substitute the core proteins and be modified in similar ways. The core histone H2A has the most identified variants, including H2A.X, H2A.Z, and macroH2A (Chadwick et al, 2001). MacroH2A is of particular interest due to its role in X-inactivation.

MacroH2A is a 42 kilodalton protein that appears in one of every 30 nucleosomes in a cell. It has a three part organization that includes an N-terminal H2A-like histone-fold, a non-histone region and a C-terminal highly structured globular region (Ausio et al, 2006). The histone portion of macroH2A shares 65% amino acid identity with H2A, which enables it to replace the conventional H2A. The non-histone region (NHR) or 'macro' domain, which makes up two-thirds of the protein, distinguishes macroH2A from other core histones. The NHR follows the H2A portion of the protein and includes a short linker region, a leucine zipper motif and a basic region where DNA can bind (Figure 2). The NHR can also bind ADP-ribosylate metabolites (Ausio et al, 2006).

MacroH2A has many isoforms that, although quite similar in structure, are distinct in function and expression. The two main isoforms are macroH2A1 and macroH2A2. These isoforms share 80% amino acid identity, but map to different chromosomes (Chadwick et al, 2001). The human macroH2A1 gene maps to chromosome 5 and human macroH2A2 maps to chromosome 10. They share the most similarities in the histone region and the C-terminus, indicating that these residues may be necessary for common functions of both proteins (Costanzi et al, 2001). Both isoforms

localize to the inactive X chromosome during X-inactivation, indicating a redundancy of function. However, macroH2A1 and macroH2A2 differ from each other because of non-identical distributions in nuclei (Costanzi et al, 2001). This redundancy complicates targeting a specific gene in studying the activity of this protein.

MacroH2A1 has also been found to have more isoforms that result from alternate splicing. The mouse macroH2A1 gene is found on chromosome 13 and encodes macroH2A1.1 and macroH2A1.2, which result from alternate splicing of this transcript (Figure 3). These two variants differ only by a short amino acid segment in the non-histone region, but they vary greatly in their function. The difference in structure is a result of variations in the ligand-binding region of the protein, which enables macroH2A1.1 to bind NAD metabolites while macroH2A1.2 cannot bind nucleotides (Ausio et al, 2006). These isoforms also differ in expression levels. MacroH2A1.2 has a constant level of expression whereas macroH2A1.1 has been shown to be active only during differentiation and development. MacroH2A1.2 is very similar to macroH2A2 (Constanzi et al, 2001) and preferentially associates with the inactive X chromosome (Rasmussen et al, 1999).

Male and female cells have different levels of genetic material due to the difference in the genetic content of the X and Y chromosomes. Dosage compensation, a process first identified in *Drosophila*, takes place to account for this genetic difference and maintain transcriptional balance. In mammals, dosage compensation occurs by the silencing of one of the two X chromosomes in somatic female cells (Lucchesi et al, 2005). In marsupials, X-inactivation occurs on the paternal chromosome, but in placental organisms the process is random and either paternal or maternal copies are inactivated



(Boumil et al, 2001). Much research focuses on random X-inactivation exhibited in human cells during differentiation of embryonic stem cells.

The process of mammalian X-inactivation is divided into five steps: counting, choice, initiation, spreading and maintenance. During the counting step, a cell measures the number of X-chromosomes in relation to the haploid autosome set and then chooses all but one X-chromosome to inactivate. The silencing process is then initiated and the chosen X-chromosome is inactivated. Silencing is initiated by Xist upregulation and maintained through the synergistic effects of Xist expression, DNA methylation and histone deacetylation. DNA methyltransferases have been shown to play important roles in both initiation and maintenance (Ma et al, 2005).

X-inactivation is controlled by the X-inactivation center (*XIC*), an endogenous native locus that contains the genes needed for X-inactivation. Xist, a non-coding RNA gene in the *XIC*, is essential to the silencing of the inactivated X-chromosome. It is required for initiation and establishment, but is not required for maintenance. Studies show that Xist must be activated within a narrow window of development, within 48 hours of differentiation, to work properly (Boumil et al, 2001).

MacroH2A is highly enhanced on the inactivated X chromosome. Studies indicate that macroH2A works closely with Xist during random X-inactivation in female cells. Chromatin immunoprecipitates of macroH2A1.2 contain Xist RNA, showing that Xist and macroH2A exist within the same ribonucleoprotein complex. Deposition of macroH2A on the X-chromosome is also disrupted by a decrease in Xist expression, showing their interdependence (Boumil et al, 2001).

MacroH2A is expressed in both male and female cells, but varies in localization. It has generalized nuclear distribution in male cells whereas it accumulates in a dense structure called a macrochromatin body (MCB) in female nuclei (Chadwick et al, 2001). In mouse embryonic stem cells, macroH2A is localized near the centrosome prior to X-inactivation in both female and male cells. It is believed that it is stored here until X-inactivation occurs when macroH2A1.2 relocates to the inactivated X on the seventh day of differentiation in female cells (Boumil et al, 2001).

The direct role of macroH2A in X-inactivation is unclear. The localization of macroH2A with the inactive X-chromosome does not occur until silencing has already begun, eliminating a role for macroH2A in the initiation step. However, macroH2A has a potential role in the spreading and maintenance stage of the X-inactivation process. A recent study proposed that macroH2A works by either interfering with transcription factor binding or by disturbing nucleosome remodeling. The transcription factor NF- $\kappa$ B cannot bind chromatin assembled with histone octamers containing macroH2A1.2 because of its non-histone region. Nucleosomes containing macroH2A1.2 have also been shown to resist gene promotion activity by inhibiting nucleosome remodeling (Lucchesi et al, 2005).

MacroH2A has also been linked to the cell cycle of somatic cells. A recent study found that macroH2A1 and macroH2A2 associate with centrosomes in both male and female cells and they both concentrate in distinct bands on the inactivated X chromosome in human and mouse cells (Chadwick et al 2002). However, macroH2A localization changes throughout the somatic cell cycle. MacroH2A association with the MCB is most prominent during the S phase and dissipates during the late S phase and G2 phase, before

reforming in G1. MacroH2A centrosomal localization increases as cells enter the M phase. Targeting to the centrosome appears to be part of a degradation pathway as macroH2A accumulates at the centrosome when 20S proteasome is inhibited. Alteration to macroH2A levels throughout the cell cycle is an interesting phenomenon that has never before been examined in embryonic stem cells. The goal of this study is to determine if changes seen in macroH2A during the somatic cell cycle of fixed human and mouse cells described previously hold true in the cell cycle of live mouse embryonic stem cells. It is hypothesized that macroH2A will exhibit changes during the cell cycle of mouse embryonic stem cells.

## ***Materials and Methods***

### *Cell Culture*

The female mouse embryonic stem cell lines, F121, F121-GFP and F121-GFP::macroH2A, and the male mouse embryonic stem cell line, J1, J1-GFP and J1-GFP::macroH2A were provided by Borko Tanasijevic. Plasmids encoding macroH2A::GFP fusion protein was transfected using a pEF1 vector with EGFP::mH2A1 fusion protein according to standard protocol (Figure 4) and Western Blotting was used to confirm that the cell lines expressed the transgene.

The cells were grown under standard conditions on irradiated fibroblast feeder layers in embryonic stem (ES) cell medium on gelatinized plastic plates. The ES media consisted of Dulbecco's Modified Eagle's Media (DMEM) enriched with 15% FBS, penicillin/streptomycin, non-essential amino acids, 2 $\mu$ L/L  $\beta$ -mercaptoethanol, Geneticin (G418) and 500 units/ml leukemia inhibitory factor (LIF). Feeder cells were removed by trypsinization and 30 minute incubation on plates not treated with gelatin. ES cells were

then plated without feeder cells in 1000 units/ml LIF, until all feeder cells were removed, assuring no interference with final results. Cells were passaged every 2-3 days when 10 cm dishes became confluent.

### *Microscopy*

Expression of the macroH2A::GFP construct and localization within the cell were examined using fluorescence microscopy with an Olympus Inverted Scope.

### *Cell Cycle Analysis*

Fluorescence activated cell sorting (FACS) was used to view macroH2A levels and cell cycle. MacroH2A was detected using GFP and cell cycle was examined through DNA content. All FACS was performed in the Flow Cytometry and Confocal Microscopy Facility of UConn Biotech Center using a BD FACS Aria II and Becton Dickinson FACS Calibur.

Cells were initially assayed for optimal Hoechst stain conditions, varying incubation time, temperature and staining concentration. The optimal conditions were then used to simultaneously view macroH2A and DNA content using FACS. However, proper cell cycle histograms could not be obtained using this method and a new method was determined.

Cells were sorted according to differences in GFP expression into 2% serum. Results were gated to eliminate dead cells, debris and doublets. Populations were determined according to high, medium and low expression. The sorted populations were then fixed with EtOH, stored at -20 degrees Celsius over night and stained with

propidium iodide (40ug/ul, 1 hr, covered on ice). DNA content was determined using FACS again.

Treatment with Triton X-100 was used to help improve PI staining and cell cycle analysis. Cells were sorted into 2% serum, treated with 0.2% Triton X-100, RNAase and 25 ug/ul propidium iodide and incubated in the dark for 30 minutes at RT. Cell cycle analysis was then performed using FACS.

## ***Results***

### *Expression of macroH2A::GFP Construct*

Plasmid constructs that differed in reading frames (A or B) or GFP termini (N- or C- termini) were screened in J1 cells using Western Blotting (Figure 5). C-terminal constructs with either reading frame A or B (C-A and C-B) were not expressed in these cells. N-terminal constructs with reading frame A (N-A) were also not expressed in J1 cells. However, N-terminal constructs with reading frame B (NB+) were expressed as seen by the presence of two bands, a 40 kDa band for endogenous macroH2A and a 60 kDa band for the macroH2A::GFP construct. An N-terminal construct with reading frame B that lacked GFP was used as a negative control and only expressed the endogenous macroH2A, showing that NB+ worked properly. NB+ was used to transfect all other cell lines, including the F121 cell line.

### *MacroH2A Nuclear Localization*

Microscopy was used to visualize GFP expression by F121 cells and localization of macroH2A within the cell. Phase contrast microscopy images show colonies of F121-GFP::macroH2A and F121-GFP cells. Fluorescence microscopy images show that cells

transfected with GFP alone (F121-GFP) exhibit GFP expression throughout the cytoplasm. However, cells transfected with the GFP::macroH2A (F121-GFP::macroH2A) construct exhibit more specific localization, as GFP expression is more concentrated within particular portions of the colonies (Figure 6). Although higher resolution and magnification is necessary for confirmation, these images indicate that macroH2A may be localized to the nucleus.

#### *Cell Cycle Analysis using Hoechst Staining*

MacroH2A and DNA content were simultaneously examined using GFP and Hoechst staining. After initial attempts to perform this experiment failed, an assay for optimal conditions for staining was performed, varying temperature, concentration and incubation time. Samples were first plotted forward versus side scatter and gated to eliminate dead cells, debris and double discriminates. 10 uM Hoechst stain for 45 minutes at 37 degrees Celsius, 20 uM Hoechst stain for 30 minutes at 37 degrees Celsius and 50 uM Hoechst stain for 30 minutes at room temperature, produced cell cycle histograms with unexpected peaks. 10 uM Hoechst stain for 30 minutes at 37 degrees Celsius produced the most accurate cell cycle histogram and was determined to be the optimal condition (Figure 7). These conditions were used to simultaneously look at GFP and DNA content in F121 cells. Samples were plotted forward versus side scatter and gated to eliminate dead cells, debris and double discriminates and histograms created. However, expected peaks in the cell cycle histograms were not obtained, even in the GFP only control (Figure 8). Therefore, a new method of examining this relationship was needed.

### *Cell Cycle Analysis Using Propidium Iodide*

Because Hoechst staining was not an effective method for cell cycle analysis in F121 cells, a new method using propidium iodide was developed. Cells were first sorted for high or low GFP expression using FACS, stained with propidium iodide and then analyzed for DNA content using FACS again. Three distinct populations could be seen in the F121 cells after they were gated for only GFP expressing cells. The cells were sorted according to high, medium or low GFP expression. The low population was eliminated because of low cell number. The histogram for the unsorted control stained with propidium iodide showed the expected cell cycle histogram with proper peaks, showing that this method was effective (Figure 9, 10). There were no significant differences in cell cycle histograms for GFP-only controls (Figure 9). However, distinct differences in DNA content could be seen between the populations expressing high or low GFP in the GFP::macroH2A cells. The population expressing high GFP content was mostly in the G2/M phase and the population expressing low GFP was mostly in the G1 phase (Fig 10).

Attempts to replicate these results have been difficult and treatment with 0.2% Triton X-100 was used to improve results. When experiments were repeated, differences in cell cycle phase could still be seen between the different populations and similar correlations between macroH2A and cell cycle can be seen. However, these differences are not as distinct as the original results (Figure 11). This time cells were sorted into high, medium and low expressing populations, treated with triton, stained with propidium iodide and DNA content analyzed. The medium and low populations have similar cell cycle histograms, with a high G1 peak. The high GFP population has a markedly smaller G1 peak and slightly bigger G2 peak than the medium and low GFP populations.

Cell cycle analysis was also done in the male, J1 cell line. Similar cell cycle histograms were obtained for the male cells as the female cells. The high GFP population has more G2 cells than the low and medium populations, which have more G1 cells (Figure 12). These results also show that there does not appear to be a distinct difference between male and female cells in terms of macroH2A alterations during the stem cell cycle at this stage of development (Figure 11, 12).

## ***Discussion***

MacroH2A is a core histone variant that plays a key role in understanding epigenetics controlling stem cell development and differentiation, especially during the process of X-inactivation. Its role in the maintenance stage of X-inactivation has been studied in human and mouse somatic cells (Chadwick, 2002) through the study of its distribution through the cell cycle. MacroH2A is altered during the somatic cell cycle. MacroH2A association with the MCB is most prominent during the S phase and dissipates during the late S phase and G2 phase, before reforming in G1. MacroH2A centrosomal localization increases as cells enter the M phase (Chadwick, 2002).

This goal of this study was to determine if macroH2A changes throughout the cell cycle of embryonic stem cells. Female and male mouse embryonic stem cells that were stably transfected with GFP::macroH2A were used to examine this association, using FACS. Fluorescence microscopy showed that the construct was being expressed by the cells and that the macroH2A is properly localized to the nucleus (Figure 6).

Initial problems with DNA staining in live cells using Hoechst, led to the need to develop a new method of cell cycle analysis (Figures 7, 8). However, sorting cells first



for high and low GFP expression and then staining with PI proved to be an effective method (Figure 9, 10).

Preliminary results indicate that there is an association between macroH2A and the stem cell cycle (Figure 10). Cells that were expressing high levels of GFP were mostly in the G2/M phase, while those expressing lower GFP were mostly in the G1 phase. However, attempts to replicate these findings have been unsuccessful. Although differences can be seen between the different populations, they are not as distinct as the initial findings (Figure 11). A difference can be seen between the cell cycle histograms of cells expressing high GFP and those expressing low or medium levels of GFP, but unlike the initial findings there is not as large a population in G2/M in high GFP cells or in G1 in low GFP cells. There is, however, a correlation between macroH2A expression and cell cycle phase and evidence that macroH2A does change during the stem cell cycle. There also does not appear to be a difference in profiles for male and female cell lines (Figure 11, 12), indicating that differences may not be apparent this early in the developmental process.

The apparent correlation between high GFP expressing cells and the G2/M phase and low GFP expressing cells and the G1 phase is a very interesting finding. This could mean that the GFP::macroH2A construct was incorporated into the chromatin and that it is replicated with the cellular DNA during the cell cycle. In the somatic cell cycle, similar changes to macroH2A localization can be seen in these phases. During the G2/M phase, macroH2A moves to the centrosome for degradation and during G1 it localizes to the inactivated X chromosome to reform the MCB. Excess quantities of macroH2A may be required during the G2/M stage to be targeted for degradation (Chadwick, 2002).

Although macroH2A is consistently localized to the centrosome in embryonic stem cells prior to differentiation and X-inactivation (Rasmussen 2000), its upregulation during the G2/M phase may be reflective of what happens later on in development, after differentiation into somatic cells.

This study shows that macroH2A is altered during the cell cycle of mouse embryonic stem cells as it is in somatic cells. Further research is required to examine these changes. RT-PCR can be used to determine if changes to macroH2A levels are a result of changes at the protein or RNA levels. Examining changes to macroH2A during the cell cycle in different stages of differentiation would also prove interesting. Looking at the difference between macroH2A levels in male and female cells during the process of differentiation could also help further elucidate the role of macroH2A in X-inactivation. Finally, repeating these experiments in human cells could also add a lot of valuable information to this area of research.

This study and the continued study of the epigenetic roles of macroH2A are important in furthering the knowledge in an area of research where very little is known. Understanding the role of macroH2A in the development and differentiation of stem cells is important in understanding the developmental process and can help find ways to replicate this process in vitro. Further studying the association between macroH2A and X-inactivation is also important in the field of cancer research as many tumor cells fail to undergo this process and express both X-chromosomes.

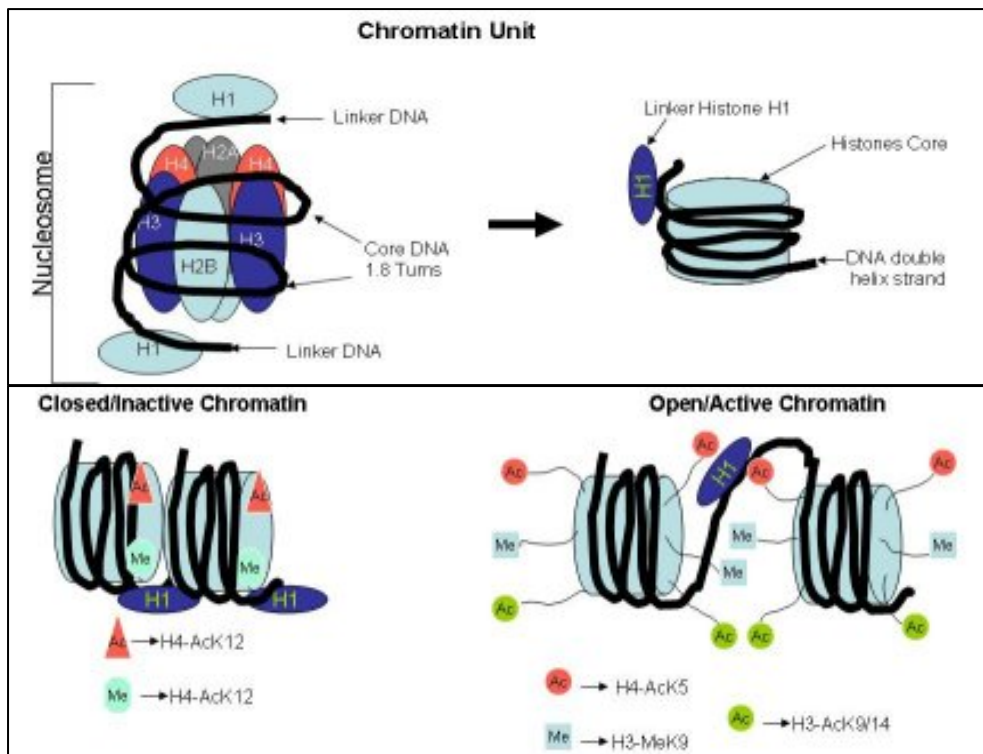
## ***Acknowledgements***

I would like to first thank Dr. Theodore Rasmussen and Borko Tanasijevic for their guidance and advice throughout the implementation of this project. Special thanks goes to Dr. Carol Norris for all her help with FACS. Thank you to Dr. Adam Zweifach for his continued support throughout the completion of this thesis. Thanks to the Honors program for the Life Sciences Research Grant to help fund this project. Thank you to Winifried Kreuger, Lindsey Swanson, and all other members of the Rasmussen Lab and the CRB for making this experience memorable. Finally, thank you to my family and friends for their continued support.

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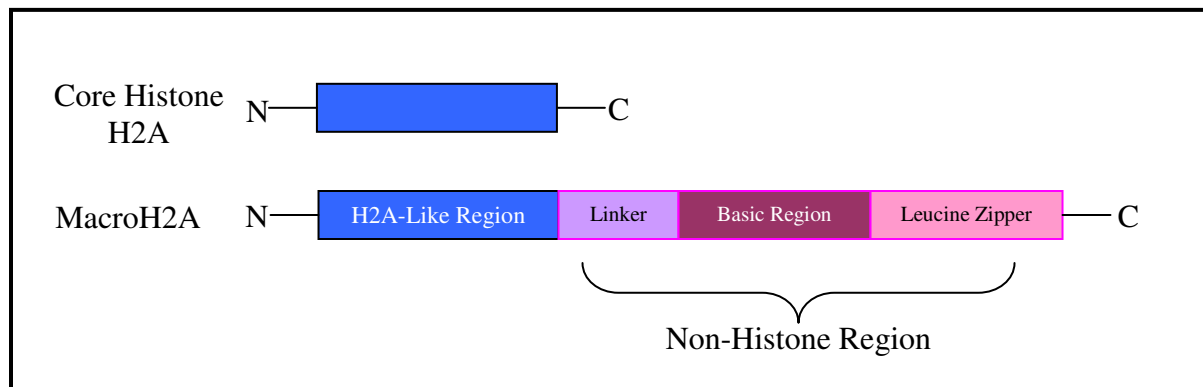
## Tables and Figures



**Figure 1:** The structure of nucleosomes and epigenetic modifications

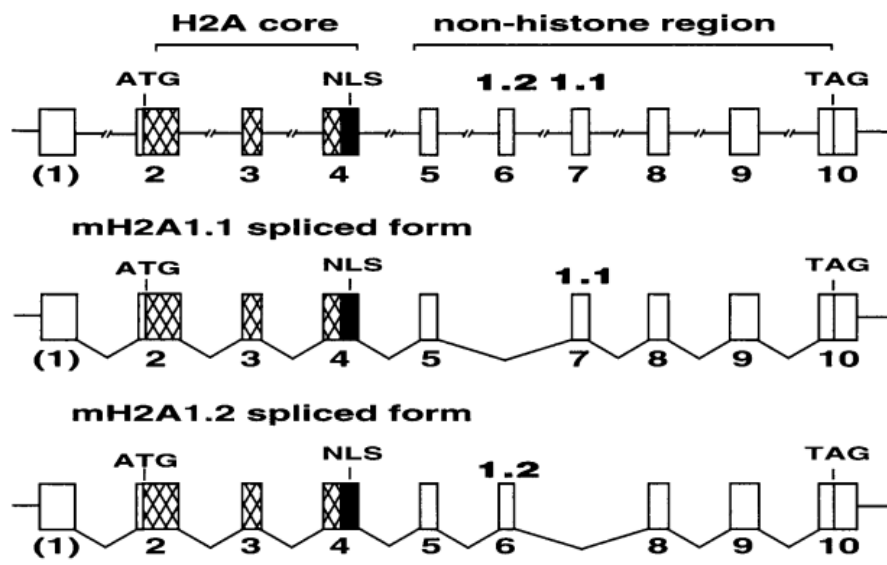
A nucleosome is composed of DNA wound around eight core histone proteins that keep the DNA compact and structured. Epigenetic modifications including methylation and acetylation, modify the DNA structure and regulate gene expression.

<http://www.abcam.com/cms/displayImage.cfm?intImageID=6801>



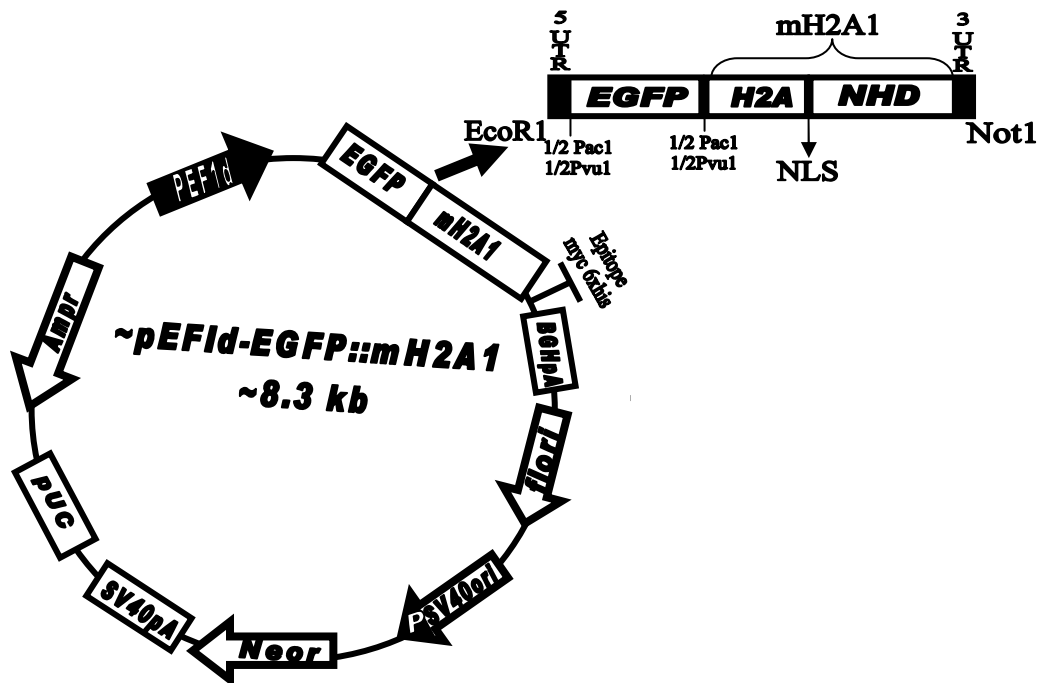
**Figure 2:** Structure of MacroH2A

MacroH2A differs from H2A in the non-histone region (NHR), which includes a short linker, leucine zipper and a basic region.



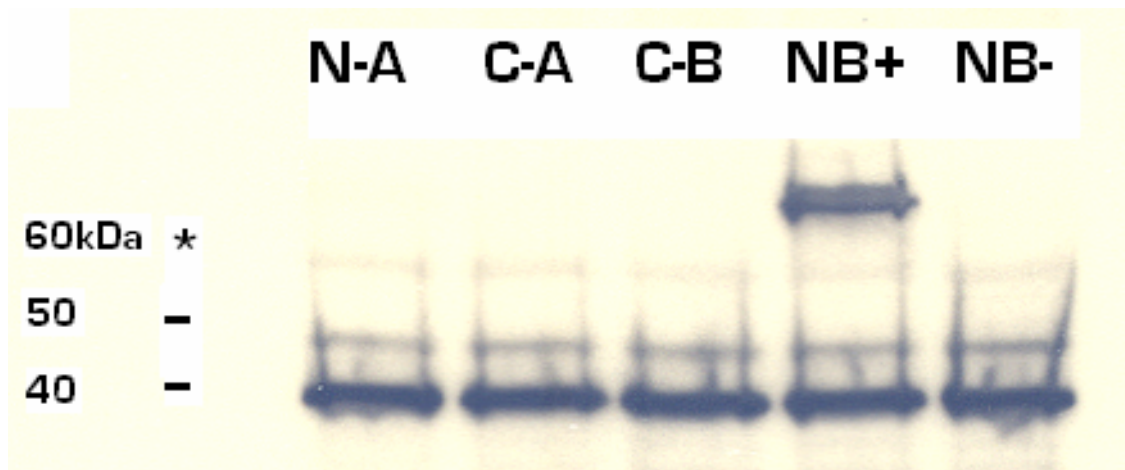
**Figure 3: Alternate splicing of macroH2A transcripts**

Adjacent but alternatively spliced exons give rise to mRNAs encoding mH2A1.1 and mH2A1.2 mature spliced mRNAs. (Rasmussen et al. 1999)



**Figure 4: MacroH2A::EGFP Vector**

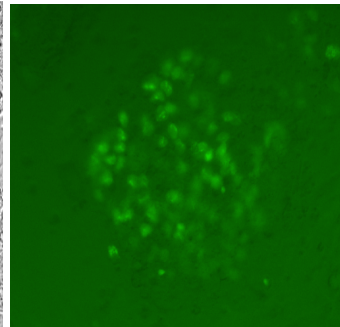
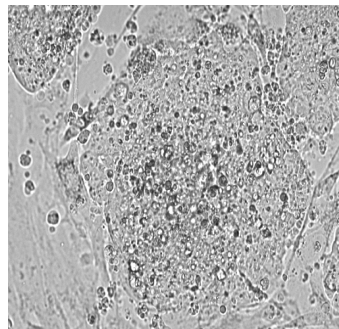
pEF1 vector with EGFP::mH2A1 fusion protein used to transfect mouse embryonic stem cells.



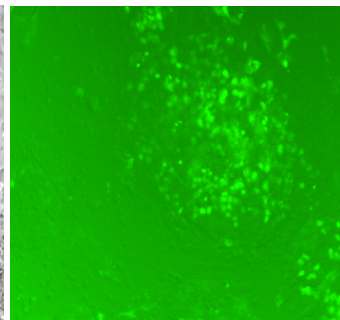
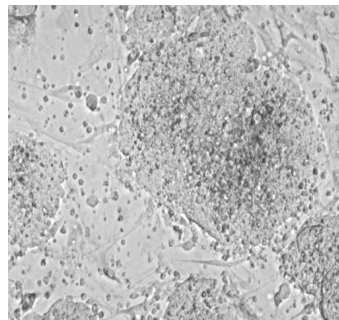
**Figure 5: Screening for Effective Plasmid**

Four constructs that differed by reading frame (A or B) and GFP termini (N or C) were screened in J1 cells for expression. N-A is an N-terminal construct with reading frame A, C-A is a C-terminus construct with reading frame A, C-B is a C-terminus construct with reading frame B. NB+ is a N-terminus construct with reading frame B and NB- is a N-terminus construct with reading frame B with out GFP, serving as a negative control. MacroH2A is a 45kDa protein and the construct a 60kDa protein.

**F121-GFP::mH2A**



**F121-GFP**

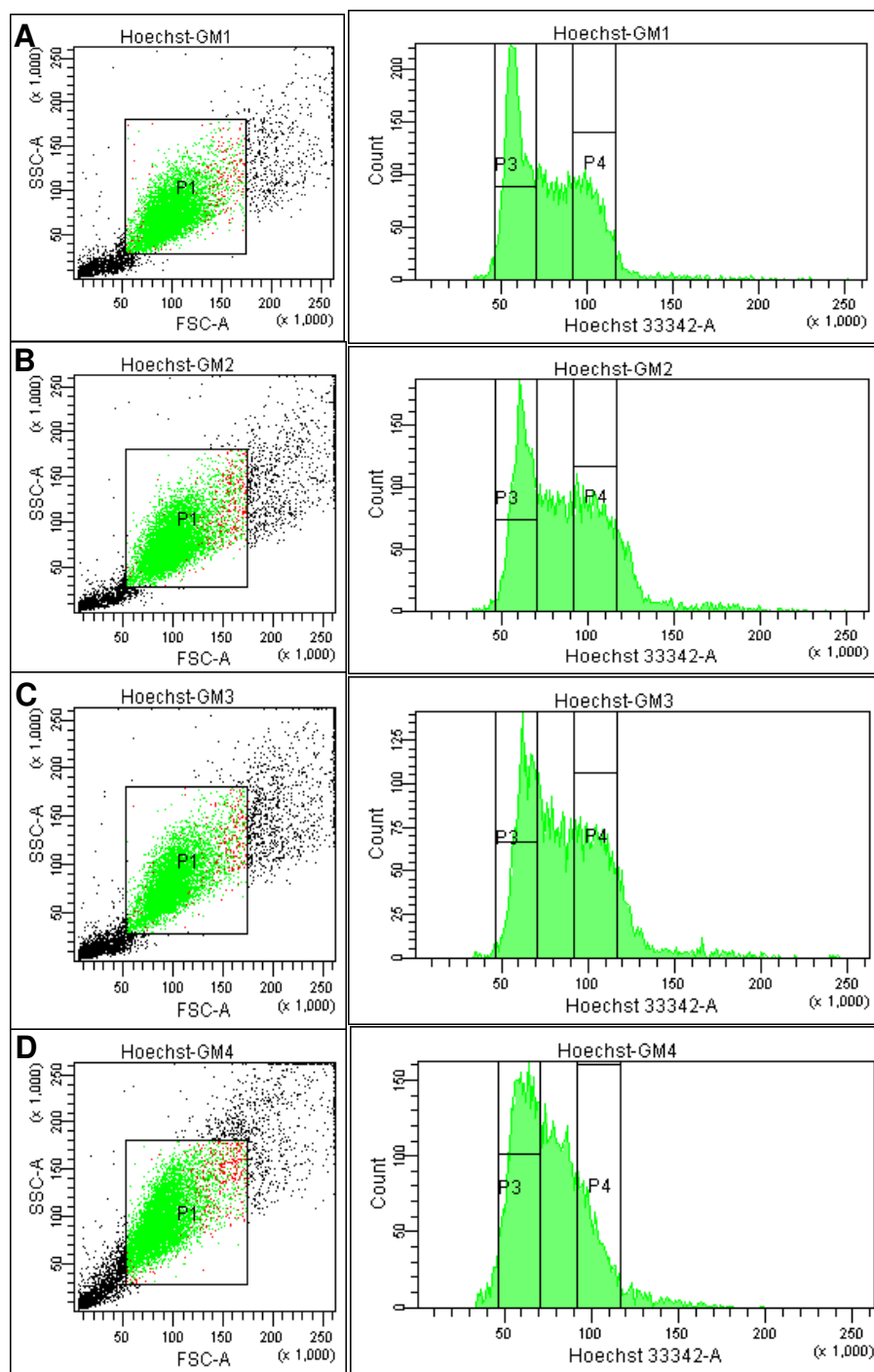


**Figure 6: F121-GFP::macroH2A and F121-GFP Cells Under Fluorescence Microscopy**

Stably Transfected Female Mouse Embryonic Stem Cells

Left Column: Phase contrast microscopy

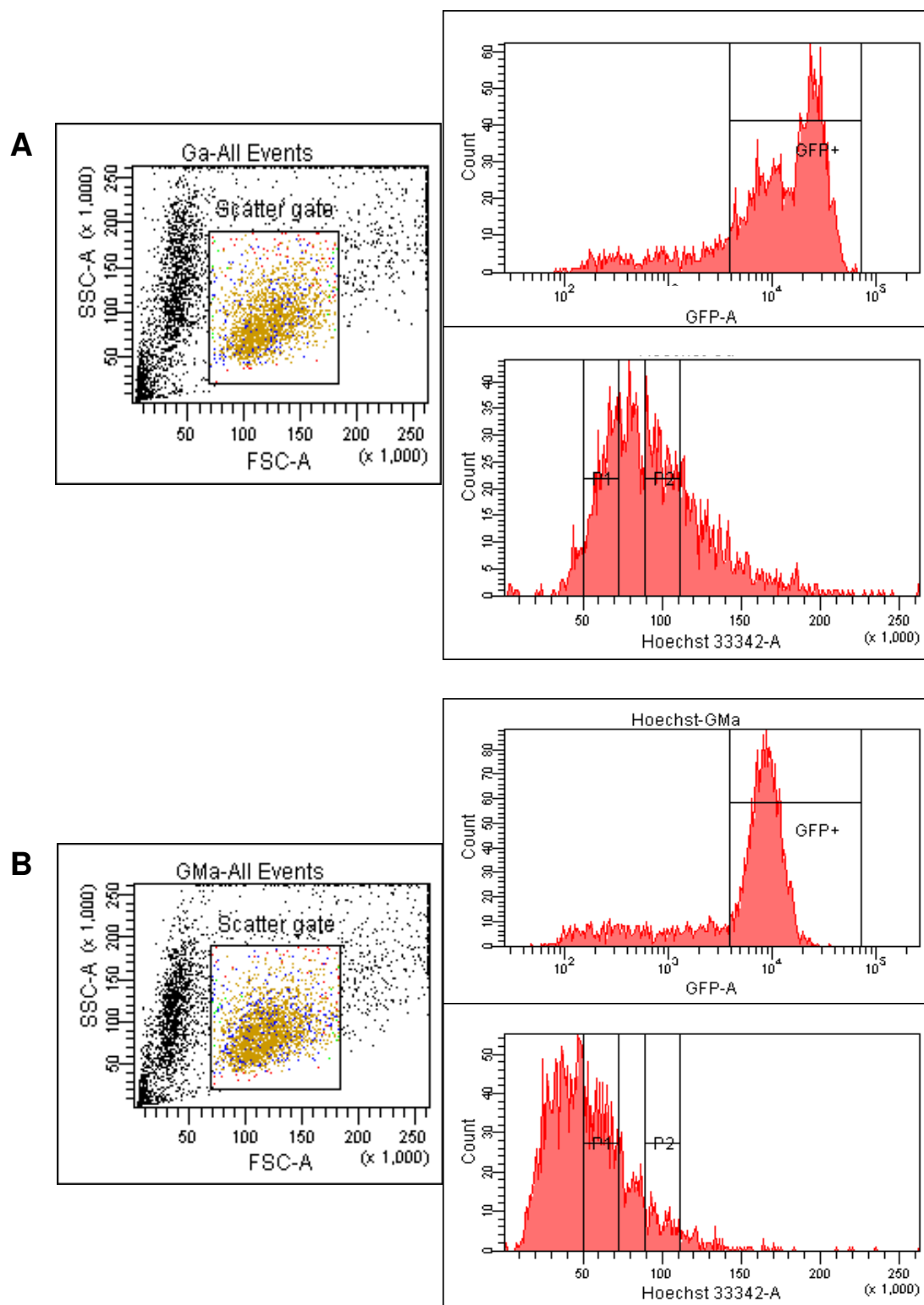
Right Column: Fluorescence Microscopy GFP positive cells



**Figure 7: Assay for Optimal Hoechst Staining Conditions**

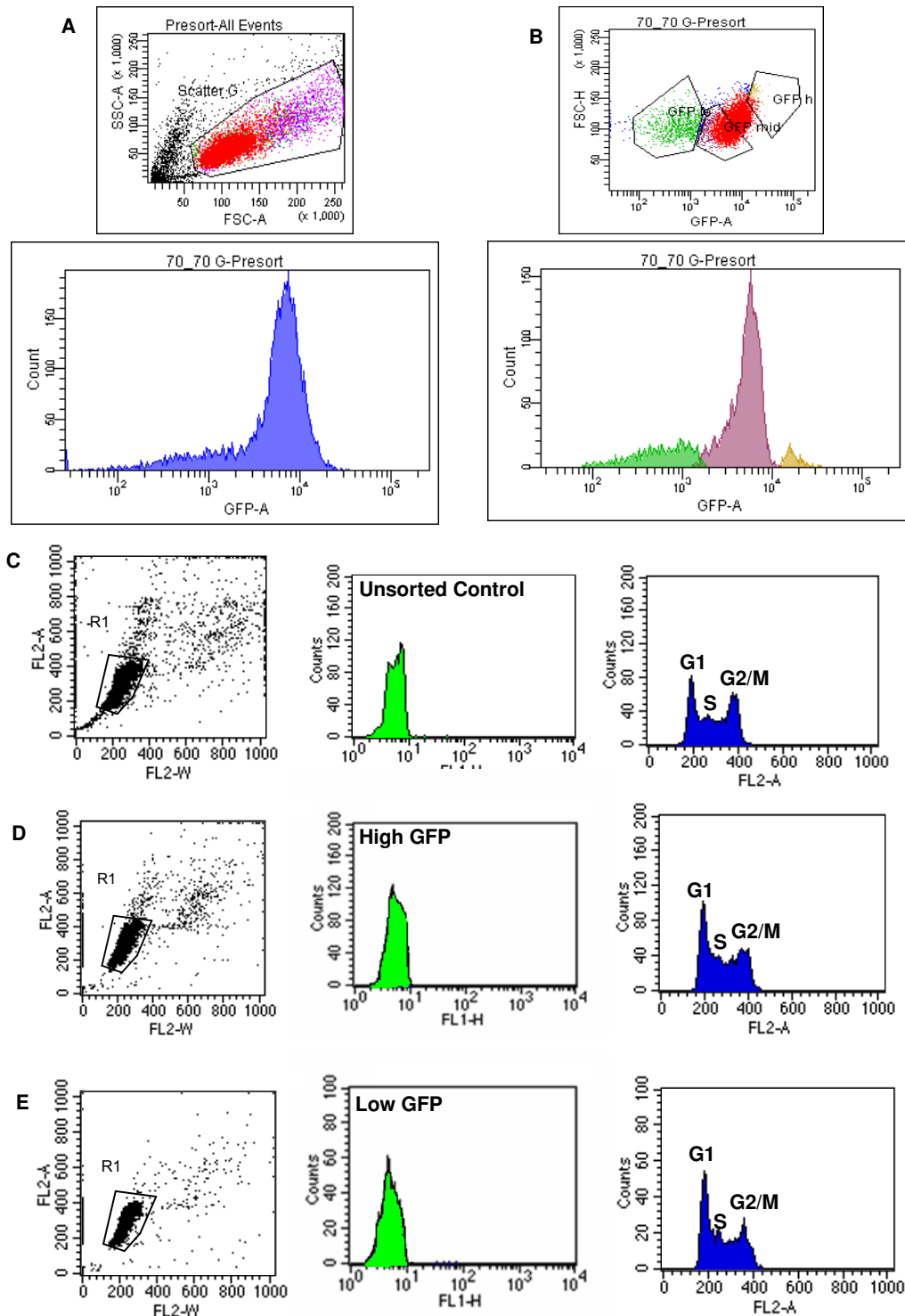
Optimal conditions for Hoechst staining was determined by varying temperature, incubation time and concentration. (A) 10 uM Hoechst Stain, 30 minutes, 37 degrees Celsius (B) 10 uM, 45 minutes, 37 degrees Celsius (C) 20 uM, 30 minutes, 37 degrees Celsius (D) 50 uM, 30 minutes at RT.





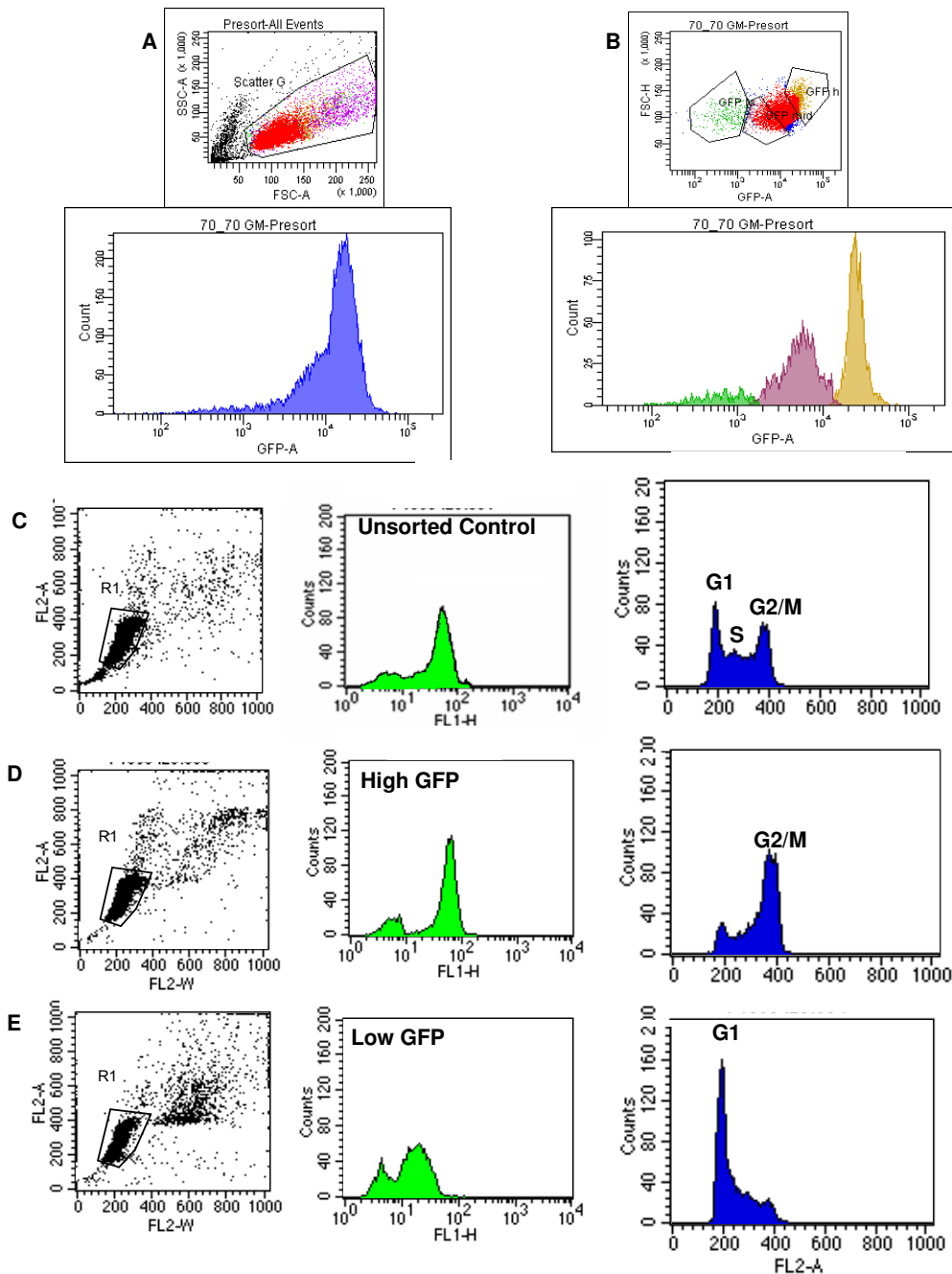
**Figure 8: Cell Cycle Analysis Using Hoechst Staining**

GFP expression and DNA were simultaneously looked at using FACS in F121-GFP (A) and F121-GFP::macroH2A cells (B).



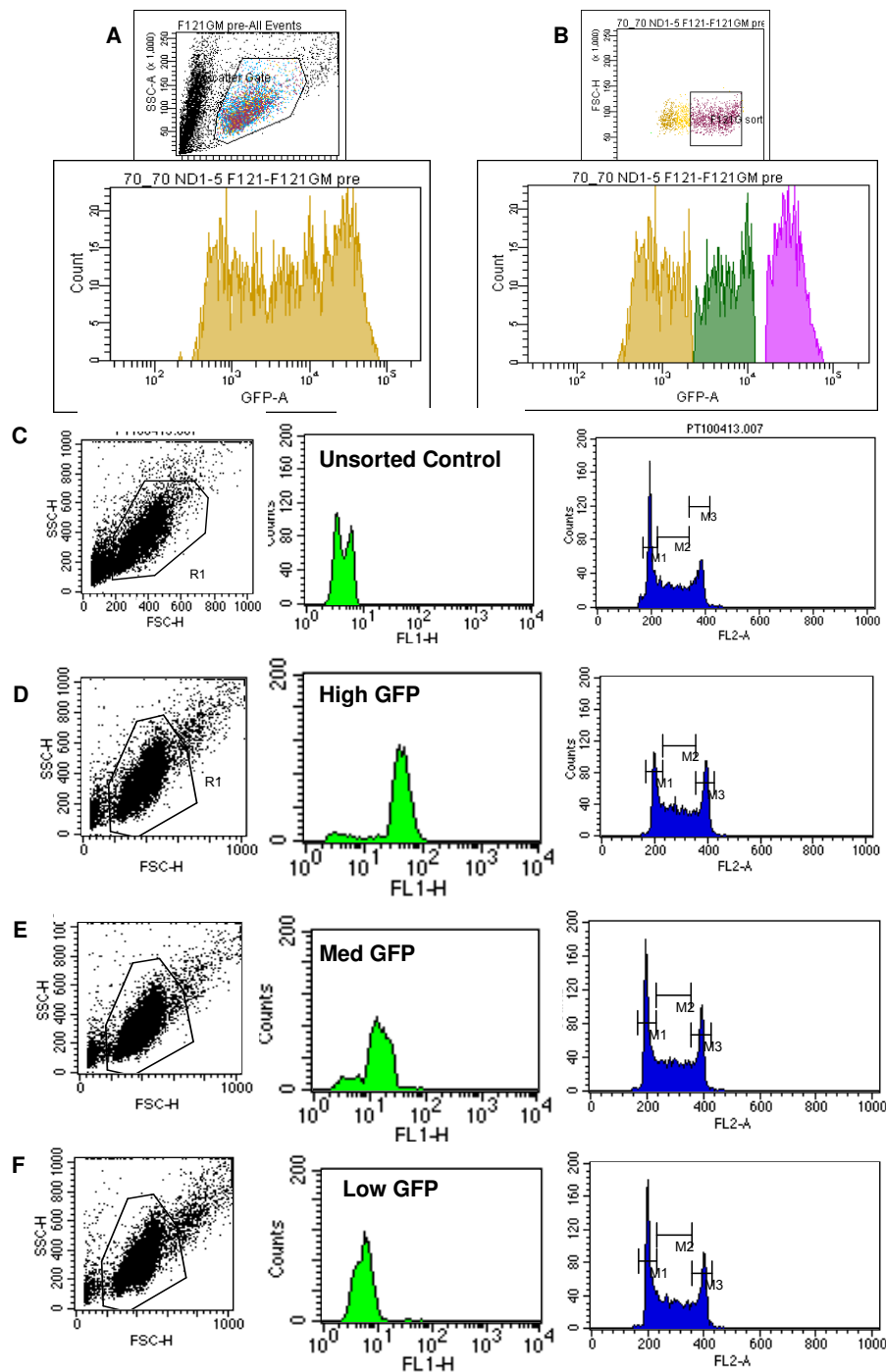
**Figure 9: GFP Control in F121 Cells.**

GFP expression was observed using FACS (A). Three distinct populations were seen and sorted (B). The lowest GFP expressing population was eliminated due to low cell number and the other two populations were fixed with EtOH and stained with PI. GFP and DNA were looked at simultaneously using FACS. Cells were gated on R1, eliminating double discriminates and debris. The cell cycle histogram for the unsorted control (C), the high GFP expressing population (D) and the low expressing population (E) are shown.



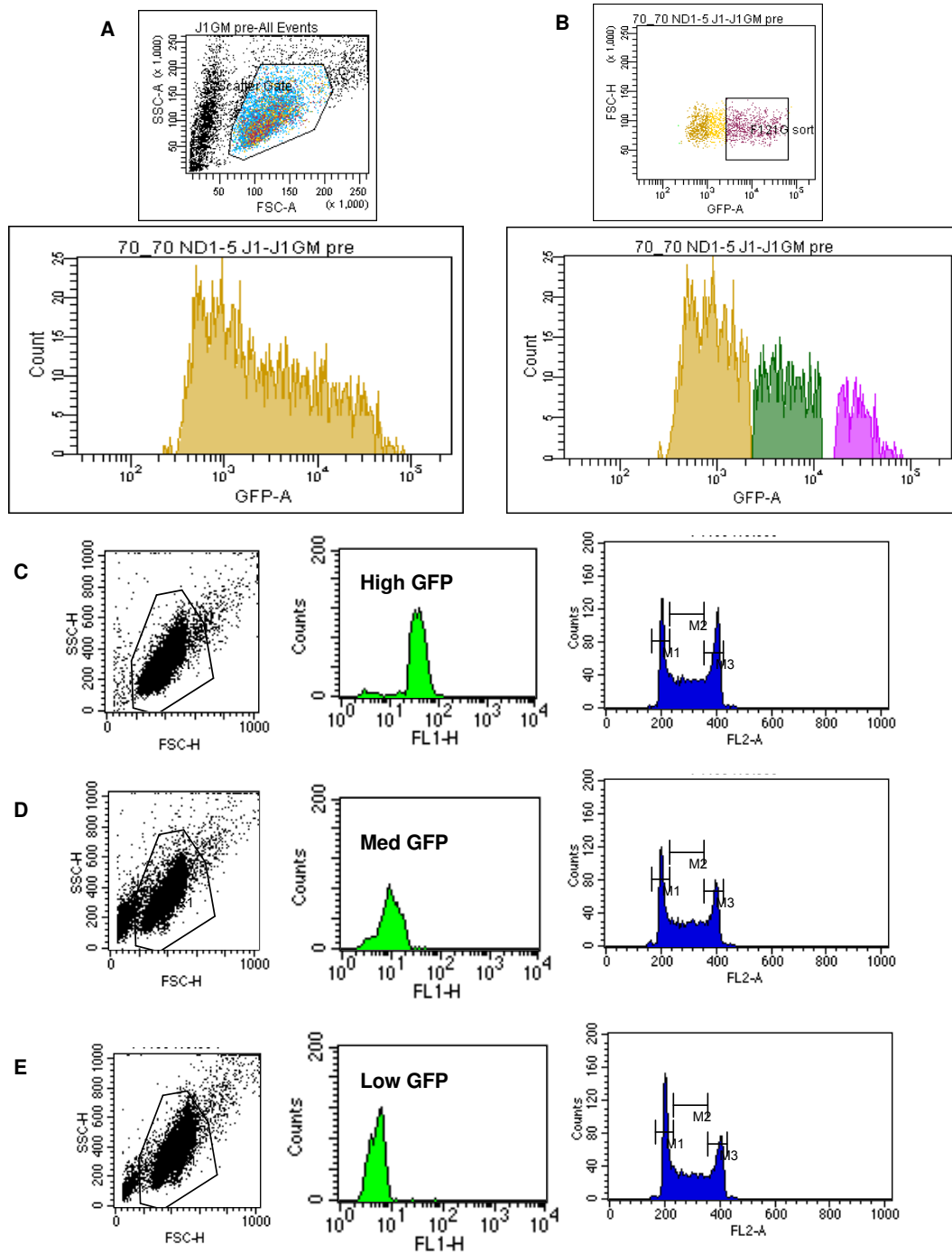
**Figure 10:** MacroH2A varies during the cell cycle in F121 Cells.

GFP expression was observed using FACS (A). Three distinct populations were seen and sorted (B). The lowest GFP expressing population was eliminated due to low cell number and the other two populations were fixed with EtOH and stained with PI. GFP and DNA were looked at simultaneously using FACS. Cells were gated on R1, eliminating double discriminates and debris. The cell cycle histogram for the unsorted control (C), the high GFP expressing population (D) and the low expressing population (E) are shown.



**Figure 11: Cell Cycle Analysis in F121 Cells**

GFP expression was observed using FACS (A). Three distinct populations were seen and sorted (B). Populations were sorted, treated with triton and stained with PI. GFP and DNA were looked at simultaneously using FACS. Cells were gated on R1, eliminating double discriminates and debris. The cell cycle histogram for the unsorted control (C), the high GFP expressing population (D), the medium expressing population (E) and the low expressing population (F) are shown. M1, M2 and M3 represent G1, S and G2/M peaks, respectively.



**Figure 12: Cell Cycle Analysis in J1 Cells**

GFP expression was observed using FACS (A). Three distinct populations were seen and sorted (B). Populations were sorted, treated with triton and stained with PI. GFP and DNA were looked at simultaneously using FACS. Cells were gated on R1, eliminating double discriminates and debris. The cell cycle histogram for high GFP expressing population (C), the medium expressing population (D) and the low expressing population (E) are shown. M1, M2 and M3 represent G1, S and G2/M peaks, respectively.