Dna damage response and repair mechanisms

Environment, Air

The stability of the genome is constantly under attack from both endogenous and exogenous DNA damaging agents. These agents, as well as naturally occurring processes such as DNA replication and recombination can result in DNA double-strand breaks (DSBs). DSBs are potentially lethal and so eukaryotic cells have evolved an elaborate pathway, the DNA damage response, which detects the damage, recruits proteins to the DSBs, activates checkpoints to stall cell cycle progression and ultimately mediates repair of the damaged DNA. As the DSBs occur in the context of chromatin, execution of this response is partly orchestrated through the modification of the DNA-bound histone proteins. These histone modifications include the addition or removal of various chemical groups or small peptides and function to change the chromatin structure or to attract factors involved in the DNA damage response, and as such, are particularly important in the early stages of the DNA damage response.

DNA double-strand breaks are repaired by different mechanisms, including homologous recombination and nonhomologous end-joining. DNA-end resection, the first step in recombination, is a key step that contributes to the choice of DSB repair. Resection, an evolutionarily conserved process that generates single-stranded DNA, is linked to checkpoint activation and is critical for survival. Failure to regulate and execute this process results in defective recombination and can contribute to human disease.

First of all, when DNA is damaged, the cell needs to stop dividing so it can heal itself and stop further spreading of the mutation. When DNA is damaged, cell cycle checkpoints are activated. There are checkpoints at G1/S and G2/M boundaries, and there is also an intra-S checkpoint. Two

kinases, ATR and ATM, controll the checkpoint activation. ATR responds when replication forks are damaged, while ATM detects double-strand breaks and mutations in chromatin structure. These kinases phosphorylate downstream targets in a signal transduction cascade, which causes the cell to pause the dividing process. The checkpoint proteins BRCA1, MDC1, and 53BP1 are probably the proteins needed to transmit the signal to activate checkpoints to downstream proteins. The checkpoint activations send out a message that the DNA needs to be repaired. DNA repair pathways will then try to repair the damage. There are different types of DNA repair mechanisms.

A system used to repair DNA is the SOS response. It involves the RecA protein and the repressor enzyme LexA. Under normal circumstance, SOS genes are negatively regulated by LexA. When DNA damage takes place, the double-stranded DNA divides into two strings in order to be able to be repaired. When there is single-strand DNA in a cell, RecA is activated. RecA inactivates LexA. When the amount of LexA decreases, the repression of SOS genes becomes less, according to the level of LexA affinity for the SOS boxes (operator sequences). Some SOS boxes bind LexA weakly, so they are free to work first. Because not every SOS box binds LexA equally strong, LexA and RecA therefore can control and activate different repair mechanisms. The first repair mechanism to be induced is nucleotide excision repair (NER). There will be more information about NER later on. When the DNA is repaired, the amount of single-strand DNA decreases. This deactivates RecA, which therefore stops deactivating LexA. LexA binds to the SOS boxes again, and the normal situation is restored.

The SOS response is very common in the bacteria domain, for example in Escherichia coli. In E. coli, the SOS boxes are 20-nucleotide long sequences near promoters, with a palindromic structure. The structure, length and composition of SOS boxes varies a lot in different organisms, but it is always highly conserved. It contains a lot of information and is considered one of the strongest short signals in the genome.

Another type of DNA repair is direct reversal. This takes place when cells can chemically reverse the damage done to their DNA. This damage occurs in only on of the four bases, so the direct reversal mechanism does not require a template. Also, the phosphodiester backbone of the DNA is not broken. There are three types of damage that cells can repair with direct reversal.

A common type of cyclobutyl dimer, thymine dimers, can be formed when UV light radiates cells, which causes adjacent thymidine bases to form a covalent bond. Photolyase, an enzyme, is activated when energy is absorbed from blue/UV light (300-500 nm wavelength). Photolyase directly reverses the damage done by the radiation of UV light. This is called the photoreactivation process.

The second type of damage that can be repaired by the use of direct reversal, is methylation of guanine bases. The protein methyl guanine methyl transferase (MGMT) directly reverses this damage. A MGMT molecule can only be used once, so this reaction is stoichiometric and takes up a lot of energy.

The last type of DNA injuries that can be chemically reversed is methylation of the bases cytosine and adenine.

When DNA is damaged which can't be chemically reversed, there are different options. There are a few different types of repair when there's damage in only one string of the DNA. When one string is damaged but the other one is still normal, it can be used as a template. The faulty base or a bigger part will be removed, and a new part will be put back in, complementary to the other string. When one base is damaged or missing, the cell uses base excision repair (BER). BER repairs damage caused by oxidation, hydrolysis, alkylation, or deamination. A DNA glycosylase removes the damaged base, and an enzyme called AP endonuclease recognises that something is missing. AP endonuclease then cuts the Phosphodiester bond, and the missing part is resynthesized by a DNA polymerase. The nick is then sealed by a DNA ligase.

Nucleotide excision repair (NER) is a very important repair system. It can recognize and repair bulky, helix-distorting lesions. Where BER can only correct damaged bases, NER can remove a bigger part of single stranded-DNA. DNA polymerase then fills up the gap, using the other strand as a template.

Mismatch repair (MMR) resolves wrong replication or recombination, when nucleotides are mispaired.

When both strands of DNA are damaged, for example in a Holliday junction, there is no strand left to use as a template to repair the DNA. For double strand damage, other repair mechanisms have to be used.

Holliday junction

Non-homologous end joining (NHEJ), is a repair mechanism that uses DNA Ligase IV, which forms a complex with the cofactor XRCC4, to join broken nucleotides together. The enzyme DNA ligase IV does this by catalyzing the formation of an internucleotide ester bond, between the deoxyribose nucleotides and the phosphate backbone. On the single-stranded tails of the DNA ends are microhomologies, these are short homologous sequences. If these microhomologies are compatible, there usually follows an accurate repair. NHEJ is also required for V(D)J recombination, the process that changes the receptors in the immune system to B-cell or T-cell receptors. During this process, there are hairpin-capped double-strand breaks, these are joined again by NHEJ. Because of this, NHEJ is an important repair mechanism, there are also backup NHEJ pathways in higher eukaryotes. However, NHEJ isn't alway flawless. When nucleotides are lost during the break, there will be nucleotides missing when these strands are joined together again. Also, the loss of nucleotides can cause the wrong strands to be connected. This can cause harmful mutations. Overall, NHEJ is a reliable process, which is especially important before the DNA replication of the cell, when there are no templates available.

When NHEJ can't be used, MMEJ is used. MMEJ uses 5-25 base pair microhomologous sequences to align strands before joining them. In order to

align them, MMEJ deletes overhanging base pairs and inserts missing nucleotides, this causes mutations. Cells will only use this if NHEJ is unavailable, because MMEJ is less reliable.

If there is a template available, homologous recombination (HR) will be used. After DNA replication, a sister chromatid can be used, or a homologous chromosome. This repair method uses an enzymatic process nearly identical to the process used for chromosomal crossover during meiosis.

If the damage in the cell is too severe, apoptosis can be induced. Sometimes DNA damage will cause the cell to malfunction, for example when it is so damaged that the cell can't get enough information from the DNA to continue making all the necessary enzymes and other important materials. Another reason for apoptosis can be when the rate of DNA damage exceeds the ability of the cell to repair the damage.

This is also used to treat cancer. Chemotherapy or radiotherapy overwhelm the capacity of the cell to cure the injuries of DNA, causing the cell to be forced to induce apoptosis. Unfortunately, this doesn't just affect cancer cells, it also affects other rapidly dividing cells such as stem cells in bone marrow. In modern treatments, it has been tried to avoid this, by concentrating the therapeutic agent in the region of the cancer cells, or by using a medicin against a feature only the cancer cells in the body have.

When DNA repair systems don't work correctly, DNA repair disorders can develop. These diseases are very dangerous, it has been shown in studies

that animals with genetic deficiencies in DNA repair often have an increased chance to develop cancer, and a shorter life.

Below is a list of genetic diseases caused by defects in repair mechanisms.

Defects in the NER mechanism are responsible for several genetic disorders, including:

- Xeroderma pigmentosum: hypersensitivity to sunlight/UV, resulting in increased skin cancer incidence and premature aging.
- Cockayne syndrome: hypersensitivity to UV and chemical agents.
- Trichothiodystrophy: sensitive skin, brittle hair and nails.
- Mental retardation often accompanies the latter two disorders, suggesting increased vulnerability of developmental neurons.

Other DNA repair disorders include:

- Werner's syndrome: premature aging and retarded growth.
- Bloom's syndrome: sunlight hypersensitivity, high incidence of malignancies (especially leukemias).
- Ataxia telangiectasia: sensitivity to ionizing radiation and some chemical agents.

All of the above diseases are often called " segmental progerias" (" accelerated aging diseases") because their victims appear elderly and suffer from aging-related diseases at an abnormally young age, while not manifesting all the symptoms of old age.

Other diseases associated with reduced DNA repair function include Fanconi's anemia, hereditary breast cancer and hereditary colon cancer.

Wikipedia – DNA repair

DNA repair diseases are hereditary, because of mutations. There's a difference between DNA damage and mutation. DNA damage is a physical abnormality in the DNA which can be recognized by enzymes. For example single or double strand breaks. Since this damage can be recognized, it can also be repaired if there's enough undamaged material suited for copying left. However, mutations cannot be recognized by enzymes because a mutation is a change in the base sequence of both DNA strands. Mutations can survive when the cell is being replicated. DNA damages are a big source of mutation, because it causes errors in replication of the cell. Since mutations can't be repaired, they survive, and when these mutations cause a disease, this disease becomes hereditary.

DNA damages are not only the cause of mutation, but also the cause of aging. Normal cellular metabolism produces byproducts such as reactive oxygen species. These are the cause of DNA damages leading to aging and loss of functional capacity when aging. A calorie-restricted diet causes less reactive oxygen species and increases the life span of mammals. This indicates that oxidative DNA damage in a cause of aging.

Overall, DNA damages cause a lot of trouble, and therefore repair mechanisms are very important for survival and evolution.

Background information
Ubiquitin

Ribbon representation of ubiquitin. Molecular surface of ubiquitin.

Ubiquitin is a regulatory protein that can be found in every cell in eukaryotes. Ubiquitination refers to the modification after translation of a protein by the covalent bond of one or more ubiquitin monomers. The most important function of ubiquitin is labeling proteins for proteasomal degradation. Besides this function, ubiquitination also controls the stability, function, and intracellular localization of a wide variety of proteins.

The ubiquitylation system

Ubiquitin (originally, Ubiquitous Immunopoietic Polypeptide) was first identified in 1975 as a protein with an unknown function which was found in every living cell. The basic functions of ubiquitin and the ubiquitination pathway were discovered in the early 1980s for which the Nobel Prize in Chemistry was awarded in 2004.

The destruction of proteins is as important as their synthesis for the maintenance of protein homeostasis in cells. In eukaryotes, the ubiquitin-proteasome system is responsible for a large part of this protein breakdown: the small protein ubiquitin tags and targets other proteins to go to the proteasome.

With the discovery in the late 1980s that the DNA-repair gene RAD6 encodes a ubiquitin-conjugating enzyme, it became clear that protein modification through a bond with ubiquitin has a much bigger impact than anyone had thought before. These days, ubiquitinis implicated in a range of human diseases, including breast cancer and Fanconi anaemia, which is very important for studies focused on the relationships between ubiquitin and

DNA-repair. Destruction with the use of ubiquitin plays a crucial part in cell-cycle regulation, DNA repair, cell growth and immune function, as well as in hormone-mediated signalling in plants. Ubiquitin has been shown to have numerous non-digestive functions, including involvement in vesicular trafficking pathways, regulation of histone modification and viral budding.

Proteins can be modified through attachment to ubiquitin and ubiquitin-like proteins. Bound ubiquitin regulates the interactions of proteins with other molecules, for example binding to the proteasome or recruitment to chromatin. The various ubiquitin systems use related enzymes to attach specific ubiquitin-like proteins to proteins, and most of these attachments are fleeting. There is a lot of evidence suggesting that the modification evolved from prokaryotic sulphurtransferase systems or related enzymes. The attachment of ubiquitin to proteins probably didn't first evolve in eukaryotes, because proteins similar to the enzymes that are involved in the attachment and disattachment of ubiquitin seem to have been formed at the time of the last common ancestor of eukaryotes.

Given the central role of the ubiquitin system in diverse cellular processes, it is not surprising that its dysfunction contributes to cancer and to severe disorders. It is important to understand the ubiquitin system to find suitable treatments for such diseases.

The Ubiquitin Proteasome Pathway has been linked to diseases involving all types of cellular activity including:

- Antigen processing

- Apoptosis

- Cell cycle and division

- DNA transcription and repair

- Immune response and inflammation

- Neural and muscular degeneration

- Viral infection

Some genetic disorders associated with ubiquitin are:

- The gene disrupted in Liddle's Syndrome results in disregulation of an epithelial Na+ channel (ENaC) and causes hypertension.

- Eight of the thirteen identified genes whose disruption causes Fanconi anemia encode proteins that form a large ubiquitin ligase (E3) complex.

- Mutations of the Cullin7 E3 ubiquitin ligase gene are linked to 3-M syndrome, an autosomal-recessive growth retardation disorder.

Immuno precipitation

Immunoprecipitation is the technique of precipitating a protein antigen out of solution using an antibody that specifically binds to the protein. This process can be used to isolate and concentrate a particular protein from a sample containing many thousands of different proteins. Immunoprecipitation requires that the antibody binds to beads that can be separated from the rest of the solution.

We've used this slightly altered technique: Protein complex immunoprecipitation (Co-IP)

Co-IP works by selecting an antibody that targets a known protein that is believed to be a member of a larger complex of proteins. By targeting this known member with an antibody it may become possible to pull the entire protein complex out of the solution and identify the proteins that are bound together.

This works when the proteins involved in the complex bind to each other tightly, making it possible to pull multiple proteins out of solution by latching onto one member with an antibody. This is called a " pull-down". It may be required to do several rounds of precipitation with different antibodies.

Repeating the experiment by targeting different members of the protein complex helps to double-check the result. Each round of pull-downs should result in the recovery of both the original known protein, in this case ubiquitin, as well as other proteins of the complex. By repeating the immunoprecipitation in this way, the researcher verifies that each identified member of the protein complex was a valid identification.

The two general methods for immunoprecipitation are the direct capture method and the indirect capture method. Both methods gives the same end-result with the protein bound to the antibodies which are on the beads.

The direct method is that antibodies that are specific for a particular protein (or group of proteins) are immobilized on a solid-phase substrate such as microscopic agarose beads. The beads with bound antibodies are then added to the protein mixture and the proteins that are targeted by the antibodies are captured onto the beads via the antibodies.

We've used the indirect method. The indirect method is that antibodies that are specific for a particular protein, or a group of proteins, are added directly to the mixture of protein. The antibodies have not been attached to a solid-phase support yet. The antibodies are free to float around the protein mixture and bind their targets. As time passes, the beads coated in protein are added to the the mixture of antibody and protein. At this point, the antibodies, which are now bound to their targets, will stick to the beads.

With our experiment the indirect method was preferred because it wasn't known if the concentration of the protein target was low or the bond of the antibody with the protein was weak.

The beads have to be separated from the rest of the sample, so that the beads can be washed to remove non-bound proteins.

This is done by spinning the sample in a centrifuge. After this step, the beads form a very small pellet at the bottom of the tube. The liquid floating above it is carefully removed to not disturb the beads. The buffer solution can then be added to the beads and after mixing, the beads are pulled down of the wash solution by centrifuging the sample again.

After that, the beads were washed several times to remove any proteins that are not bound to the antibody on the beads. After washing, the proteins were eluted and analyzed using gel electrophoresis and western blotting.

Western blot

The western blot is a technique used to find specific proteins in a sample. It uses gel electrophoresis to separate denatured proteins by the length of the

polypeptide. The proteins are then transferred to a membrane, where they are detected using antibodies specific to the target protein.

We've taken samples from cell culture. The cells were broken down by using a lysate, a solution that destroys the cell membrane.

We've used a buffer to loosen the proteins from the cell. Protease and phosphatase inhibitors are added to prevent the digestion of the sample by its own enzymes. This was done in a bucket of ice to avoid protein denaturing.

The proteins of the sample are separated using gel electrophoresis. Separation of proteins may be by isoelectric point (pI), molecular weight, electric charge, or a combination of these factors. The nature of the separation depends on the treatment of the sample and the nature of the gel.

The most common type of gel electrophoresis employs polyacrylamide gels and buffers loaded with sodium dodecyl sulfate (SDS). SDS-PAGE, polyacrylamide gel electrophoresis, maintains polypeptides in a denatured state once they have been treated with strong reducing agents to remove secondary and tertiary structure and allows separation of proteins by their molecular weight. Sampled proteins become covered in the negatively charged SDS and move to the positively charged electrode through the gel. Smaller proteins move faster through the gel and the proteins are separated by their size, which is measured in kilodaltons (kDa).

Samples are loaded into lanes in the gel. The first lane is used for the marker, a mixture of proteins with molecular weights that are known, stained to form visible, coloured bands. When voltage is applied along the gel, proteins move into it at different speeds. These different rates of movement separate into bands within each lane.

In order to make the proteins accessible to antibody detection, they are moved from within the gel onto a membrane. The membrane is placed on top of the gel, and a stack of filter papers placed on top of that. The entire stack is placed in a buffer solution which moves up the paper by capillary action, bringing the proteins with it. Another method for transferring the proteins is called blotting and uses an electric current to pull proteins from the gel into the membrane. The proteins move from within the gel onto the membrane while maintaining the organization they had within the gel. As a result of this process, the proteins are put on a thin surface layer for detection.

The overall effectiveness of transfer of protein from the gel to the membrane can be checked by staining the membrane.

The membrane has been chosen for its ability to bind protein, and both antibodies and the target are proteins, so interaction between the membrane and the antibody must be prevented for detection of the target protein. This can be done by placing the membrane in a solution of non-fat dry milk, with a little bit of Tween, a detergent. The protein in the milk solution attaches to the membrane in all places where the target proteins have not attached. This is done to make sure that there is no room on the

membrane for the antibody to attach other than on the binding sites of the specific target protein when the antibody is added. This reduces " background noise" in the final product of the Western blot, leading to clearer results, and eliminates false positives.

During the detection process the membrane is searched for the protein of interest with a modified antibody which is linked to a reporter enzyme, which when exposed to an appropriate substrate drives a colourimetric reaction and produces a colour. For a variety of reasons, this traditionally takes place in a two-step process, although there are now one-step detection methods available for certain applications.

Antibodies are generated when a host species or immune cell culture is exposed to the protein of interest. Normally, this is part of the immune response, whereas here they are harvested and used as sensitive and specific detection tools that bind the protein directly.

After blocking, a dilute solution of primary antibody is incubated with the membrane. The solution is composed of buffered saline solution with a little bit of detergent and powdered milk. The antibody solution and the membrane were sealed and incubated together for 60 minutes.

After rinsing the membrane to remove unbound primary antibody, another antibody is put on the membrane, directed at a species-specific portion of the primary antibody. This is the secondary antibody, and because of the targeting properties, it's called " anti-mouse" or another species. Antibodies come from animals; an anti-mouse secondary will bind to almost any mouse-

sourced primary antibody. Several secondary antibodies will bind to one primary antibody so the signal will be bigger.

Most commonly, a secondary antibody is used to bind a chemiluminescent agent, and the reaction product produces luminescence in proportion to the amount of protein. A sensitive sheet of photographic film is placed against the membrane, and exposure to the light from the reaction creates an image of the antibodies bound to the blot.

Another method of secondary antibody detection utilizes a near-infrared (NIR) fluorophore-linked antibody. Light produced from the excitation of a fluorescent dye is static, making fluorescent detection a more precise and accurate measure of the difference in signal produced by labeled antibodies bound to proteins on a Western blot. Proteins can be accurately quantified because the signal generated by the different amounts of proteins on the membranes is measured in a static state, as compared to chemiluminescence, in which light is measured in a dynamic state.

The colorimetric detection method depends on incubation of the Western blot with a substrate that reacts with the reporter enzyme that is bound to the secondary antibody. This converts the soluble dye into an insoluble form of a different color that precipitates next to the enzyme and thereby stains the membrane. Development of the blot is then stopped by washing away the soluble dye. Protein levels are evaluated through the intensity of the stain.

Silver Stain

Silver staining is used to detect proteins separated by gel electrophoresis.

Protein detection depends on the binding of silver ions to the amino acid side chains, primary the sulfhydril and carboxiyl groups of proteins, followed by reduction to free metallic silver. The protein bands are visualized as spots where the reduction occurs and, as a result, the image of protein distribution within the gel is based on the difference in oxidation-reduction potential between the gel's area occupied by proteins and the free adjacent sites. A number of alteration in the silver staining procedure can shift the oxidation-reduction equilibrium in a way that gel-separated proteins will be visualized either as positively or negatively stained bands. The silver amine or alkaline methods usually have lower background and as a result are most sensitive but require longer procedures. Several modifications of the silver nitrate staining procedure have been developed for visualizing proteins that can be subsequently digested, recovered from the gel, and subjected to mass-spectrometry (MS) analysis, a tool that is being used in combination with gel electrophoresis or chromatographic methods for rapid protein identification.

After electrophoresis, the gel is removed from the cassette and placed into a tray containing an appropriate volume of fixing solution. The gel is soaked in this solution overnight. This fixation will restrict protein movement from the gel and will remove interfering ions and detergent from the gel. When it's left overnight it may improve the sensitivity of the staining and decrease the background.

Then the gel is washed in 20% ethanol for 20 min. the solution three times to remove the remaining detergent ions as well as fixation acid from the gel.

Note: We recommend using ethanol solution instead of deionized water to prevent gel's swallowing. If water is used during the washing step the size of the gel can be restoring by incubation of the gel in 20% ethanol for 20 min. The ethanol solution is washed off and the sensitizing solution is added. It's incubated for two minutes with gentle rotation. This will increase the sensitivity and the contrast of the staining. The sensitizing solution is washed off and the gel is washed twice.

The cold silver staining solution is added and the testtube is shaken for 20 min to allow the silver ions to bind to proteins. After staining is complete, the staining solution is poured off and the gel is rinsed with a large volume of deionized water to remove the excess of unbound silver ions. This is repeated. If the gel is washed for more than one minute, it will remove the silver ions from the gel resulting in decreased sensitivity.

The gel is rinsed shortly with the developing solution. A new portion of the developing solution is added and the protein image is developed by incubating the gel in developing solution. The reaction can be stopped as soon as the desired intensity of the bands is reached.

The reduction reaction is stopped by adding terminating solution directly to the gel that is still immersed into developing solution. the gel is gently moved in the solution. Moist gels can be kept in 12% acetic acid at four degrees Celsius in sealed plastic bags or placed in the drying solution for 2 hours before vacuum drying, which is what we've done.

The experiments

Purpose

To optimise the technique to separate ubiquitinated proteins from the other proteins by using ubiQ beads and showing them by using antibodies. To show the efficiency of the separation by using the western blot and show the specificity by using the silver stain technique.

Introduction

The focus was on blocking the DUB enzyme, which is an enzyme that takes the ubiquitin off of the proteins. This means that the E3 enzyme, which binds the ubiquitin to the substrate, can bind all the ubiquitin to the target substrates. The ubiquitin will attach itself onto the beads. When the sample is washed, all the loose proteins will be gone from the sample. The ubiQ beads are divided in three parts which will undergo a different treatment. The first one will be boiled; the second one eluted with an acidic liquid; the third one will be eluted with SDS. These three methods will take the ubiquitin bound proteins from the ubiQ beads so the results can be measured. The proteins that weren't ubiquitinated will be used as an input sample together with the Hela lysate which has no proteins in it. The input samples are used to eliminate background static on the gel scan.

Hypothesis

A large part of the ubiquitin will bind to the ubiQ beads and will stay in the sample. This will be made visible with several different techniques. The boiled sample will have less ubiquitinated proteins, because the proteins will

denaturize because of the high temperature and a large part won't be available for further research.

Materials and methods

Urea is a powerful protein denaturant, that disrupts the noncovalent bonds in the proteins. Urea can be used to increase the solubility of some proteins.

In lysis buffers, NEM is used to inhibit deubiquitination of proteins for Western Blot analysis.

MG132 is a specific, potent, reversible, and cell-permeable proteasome inhibitor. It reduces the degradation of ubiquitin-conjugated proteins.

Immunoprecipitation

Lyse cells and prepare sample for immunoprecipitation.

Pre-clear the sample by passing the sample over beads that are not coated with antibody to soak up any proteins that non-specifically bind to the beads.

Incubate solution with antibody against the protein of interest. Antibody is attached to solid support after this step.

Continue the incubation