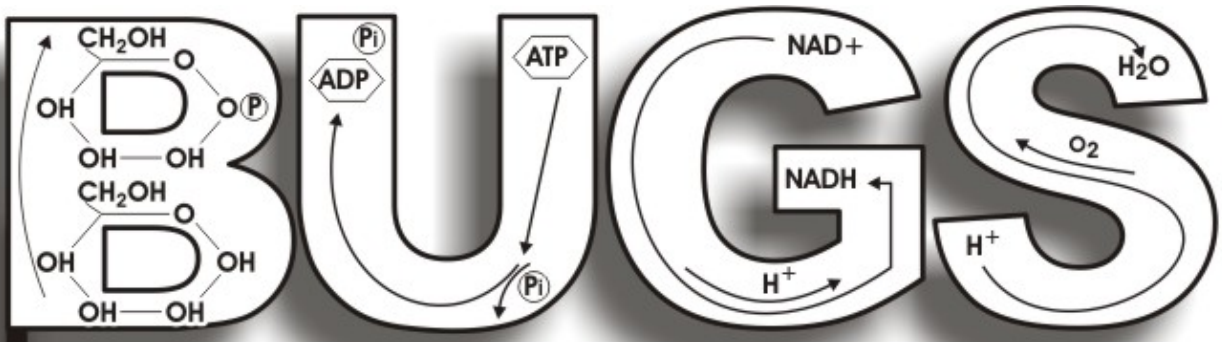
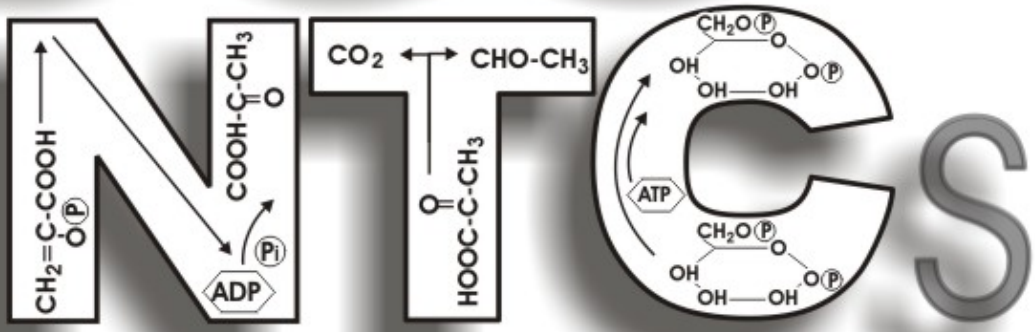




BIOC 312

SET # 7 Lectures 23-26 (03/07/07 –03/16/07)

ANNOUNCEMENTS

- BUGS elections! Campaigning from Wednesday, March 28th until Monday, April 2nd. Come out to the BUGS **Wine and Cheese on Monday, April 2nd** to meet your BUGS election Candidates.
- Returning U2 Students may apply for the BUGS scholarship worth **500$.** Applications due **Monday, April 2nd.**

< Biochemistry Undergraduate Society - 5th Floor McIntyre rm. 511 >

< bugs@sus.mcgill.ca - (514) 398-5247>

03/07/2007

Lecture #23– Apoptosis

NOTE: This NTC is meant to be used as a study aid to supplement your own class notes. Hence, not all of the text or diagrams contained in the lecture slides will be reproduced here.
Please send any comments or questions about NTCs to either Misha or James through e-mail at **bugs_u2reps@yahoo.ca**

Dr Shore was away therefore the lecture was given by Matthew War, a graduate student in Dr Shore's lab who specializes on Apoptosis.

Apoptosis:

- **Programmed cell death or apoptosis** is cellular degradation driven by proteolytic events essential for normal cell development, tissue homeostasis, and elimination of damaged or dangerous cells
- It is thought to be present in every multicellular organism and acts as a defense mechanism against a cell that is no longer useful or could provide a threat to the entire organism
- It is utilized during cell suicide by a damaged or dangerous cell to maintain homeostasis of the entire organism.
- There are distinct specific biochemical changes that cause the cell to undergo apoptosis which is very different from other types of cell death ie: necrosis.
- Necrosis is an important form of cell death. It is a nonspecific "accidental" event following cellular trauma, rather than a genetically defined and biochemically executed cellular pathway (as is apoptosis). It has hallmark features that are very different from apoptosis.
- The cell death mechanism of apoptosis was recognized in 1972 by Kerr, Wylie and Currie
    1. They published a paper describing what seemed to be a novel cell death pathway; more specifically it described the **morphology the cell displays before it dies**.
- In apoptosis the cell is taken apart and disassembled in a coordinated way so that no proteins are released into the circulation.
- The body does not see it as an injury, and thus the cell is cleared in a coordinated manner.
    1. There is **cellular condensation**. The organelles remain structurally undamaged. Chromatin becomes condensed and marginalized along one side of the nucleus.
    2. The cell membrane then starts to fragment, encapsulating the contents of the cell. Membrane bound **apoptotic bodies** are thus formed. There is no release of proteins at this stage and the organelles are in tact.
    3. Chromatin is degraded by specific cleavage and fragmentation.
- The apoptotic bodies are then phagocytosed either by neighboring cells or specialized macrophages.
- There is no remnant of the cell death pathway as there is in necrosis.

Necrosis:

- In necrosis, cells undergo trauma and swell. As a consequence, the organelles also swell and become damaged along with the cell membrane. The cell falls apart in a nonspecific way. Proteins are released into the circulation which induces a major inflammatory response by the organism (the induction of macrophages, neutrophils, leucocytes etc. attack the area to get rid of cellular debris). Cellular debris is not generally a single cell, but a patch within a tissue. **A hallmark of necrosis is inflammation**.

Caspases:

- One of the main biochemical changes in apoptosis is the activation of caspases.

- Caspases are a family of proteases (enzymes that are able to cleave proteins by hydrolysis)
    1. They are very specific and are only activated during apoptosis
        - **They are considered a marker for apoptosis**
    2. The caspases cleave protein substrates which result in morphological changes by cleaving key regulatory and structural proteins such as actin (cytoskeleton), "icad" (caspase inhibitor) and "parp" (DNA repair) which are regulatory proteins.

How can one assay for cells undergoing apoptosis?

- Because of their special apoptotic morphology; it is very easy to assay for these cells.
    - The main assay used is **"DNA laddering"**
    - This happens during apoptosis as caspases cleave "ICAD" which can no longer inactivate CAD which is free to chop up chromatin.
        - These cleave linker DNA in between histones
- An investigator can run a DNA gel electrophoresis and would note that DNA is cleaved between nucleosomes resulting in a ladder of oligo or polynucleosomes of diminishing size.

Protein Blot

- There are 300-400 proteins that are proteolytically cleaved either at one site or two sites and these can be recognized in the cell. A very simple assay for apoptosis involves running a protein gel to see if a given protein is whole or if it has been cleaved.
- Necrotic cells will show one band on a gel while apoptotic cells will show 2. The molecular weights of fragments would add up to the intact protein if the bands are the result of a cleavage product.

Phosphatidyl Serine

- The cell membrane also undergoes a biochemical change. There is a phospholipid called phosphatidyl serine (PS), which orients its phospholipid headgroups facing the inside of a healthy cell (inner half of membrane). During apoptosis PS flips and the headgroups are on the outside the cell. This is the signal for apoptotic bodies to be picked up by neighboring cells or macrophages. A fluorescent dye (Annexin V) stains for PS; healthy cells won't stain because PS is on the inside.

DNA Fluorescence

- DNA binding stains intensely fluoresce in apoptotic cells (since the DNA condenses) and this can therefore serve as yet another assay.

Mitochondrial Membrane

- During apoptosis, the outer mitochondrial membrane becomes permeabilized and inner membrane proteins are released which disrupts the mitochondrial potential across the inter membrane space generated by the electron transport chain
- If you stain with dyes that are specific for the membrane potential, we can then assay for apoptotic cells (which do not have a membrane potential).

Biological Roles of Apoptosis:

- In biology, the role of programmed cell death is genetically determined, biochemically executed and is responsible for many events during development.
- During development there are a number of processes involving tissue sculpting. In higher organisms the development of the hand involves interdigit tissue (webbing) and typically occurs in embryonic development. This interdigit tissue is eliminated by the process of apoptosis. There are specific signals during development that induce apoptosis in the interdigital cells, yet the digit itself remains intact.
- One can introduce either pharmacologically or genetically (e.g. gene knock-out) factors that inhibit apoptosis during embryonic development that can therefore inhibit these developmental processes.
- Another example is the formation of the preamniotic cavity. Specific tissues, (e.g. ectodermal cells) are selectively removed by apoptosis leaving a cavity.

- Structures can also be deleted by apoptosis (e.g. tadpoles who lose their tails when they are not needed anymore). In frogs, apoptosis is used to remove the tail structure.
- Mammalian development also involves apoptosis. In females the Mullerian duct (which gives rise to the uterus and the oviduct) is deleted by apoptosis in males. The Wolffian duct producing the Vas deferens in males is eliminated in females by apoptosis as well.
- Apoptosis also maintains tissue homeostasis. It is responsible for adjusting cell number. There is a huge amount of cell death going on every day.
- The brain is another example. 50% of all neurons which are made die by apoptosis during development. This process allows neurons to make appropriate contacts with axons. Only those neurons that make contact with axons survive. The rest die by apoptosis**.** Gene knockouts that target critical components of the execution of cell death result in a phenotype in which the brain is enlarged: neurons which should be dying are not.

The elimination of injured or dangerous cells (quality control):

- The immune system is one example. In the B and T lymphocytes, cells that have receptors that recognize foreign antigen survive, but those that don't have receptors for foreign antigens or that recognize self-antigen die by apoptosis (millions of cells die every day in the immune system).
    - Failure of these cell deaths would cause potential Auto-immune disorders.
- DNA damaging agents, UV radiation, damage cells and activates pathways that induce apoptosis (in damaged cells that can't be repaired).
- 50-70 billion cells die each day in 1 person. An average person in 1year will shed as many cells as the equivalent of the person's body weight.

How is cell death regulated?

- It turns out that almost all cells have an in tact machinery that induces apoptosis. The default pathway for most cells is death rather than survival shown by an experiment inhibiting protein synthesis with cycloheximide. This lead to cell death of all proteins tested. Cells survive because they have signals that suppress the cell death machinery, which remains switched 'off' in a healthy cell.
- Extrinsic and intrinsic signals determine whether the core death machinery is "on or off".
- Cytokines and hormones are extrinsic factors that circulate in the blood. If these are <u>survival</u> signals, cells receiving them stay alive. Other cells may receive <u>death</u> signals (DNA damage, virus infection, toxins, radiation, and death cytokines) that trigger apoptosis.
    - **Oncogenes** are another form of cellular stress. Apoptosis represents the first line of defense against cancer. Cells expressing oncogenes are programmed to die before proliferation can occur.
    - **P53** is an apoptosis activator; it senses DNA damage and could sense oncogene activation and would signal apoptosis of the damaged cells.
    - **Bcl2** suppresses apoptosis and high concentrations in the cell will block the death program and allow for illicit cell survival.
    - Extrinsic signals such as **FAS** ligand hit death receptors and signal apoptosis in the cell
- Signals are recognized by receptors on the cell surface that transduces signaling pathways in a cell to turn cell death machinery on or off (depending on the nature of the signal). Many signals inside the cell can also induce death or survival pathways (survival genes, oncogenes, metabolites, etc.)
- All of these pathways feed input into core death machinery.

How is Apoptosis Regulated

- A number of promoters and suppressors of apoptosis are involved in regulation.
- The decision to turn apoptosis "on or off" may be intrinsically regulated by the promoters and suppressors that regulate the core death machinery.

- Apoptosis is coupled with many diseases. These diseases arise when apoptosis is either inappropriately suppressed or activated (leading to inappropriate cell survival or death, respectively).

DISEASES ASSOCIATED WITH INHIBITION OF APOPTOSIS

Cancer

- It is initiated in a single cell, involving a genetic change resulting in the expression of a transforming proto-oncogene. Oncogenes are strong inducers of apoptosis because the body attempts to stop cancer cells from proliferating. Therefore there must also be a block of factors which induce cell death (inappropriate survival due to an outside stimulus or more likely, a defect in core suppressors or promoters).
- Most cancers are characterized by a second genetic change. There is a defect either in the promoter of the core apoptotic machinery (i.e.: p53 gene inactivation) or the inappropriate induction of a suppressor (Bcl2 induction). Generally, there is a genetic change to initiate the process and then another to prevent apoptosis from being activated.
    - More than 50% of all cancers have inactive p53; an apoptotic activator.

The vast majority of therapeutics developed work by inducing apoptosis. If, however, the cancer prevents apoptosis, it also prevents cells from responding to therapeutics.

Autoimmune Disorders

- Cells that recognize self-antigens survive instead of dying by apoptosis.
    - I.e.: rheumatoid arthritis

Viral Infections

- Viruses may encode suppressors of apoptosis by mimicking host regulatory mechanisms.
    - I.e.: p53 inactivation

DISEASES ASSOCIATED WITH INCREASED APOPTOSIS

AIDS

- Immune system shuts down because Helper T. lymphocytes are undergoing inappropriate cell death

Neurodegenerative Disorders

- E.g. Alzheimer's, Parkinson's, Huntington's and ALS are due to inappropriate cell death in brain neurons.

Myelodysplastic Syndromes

- Blood cells (platelets, neutrophils, WBCs) are not produced normally due to apoptotic events in the bone marrow.

Ischemic Injury

- Decrease in oxygen reaching the tissue (often caused by stroke). Apoptosis thus occurs in heart muscle and other tissues.

Toxin-Induced Liver Disease

- Alcohol, smoke, environmental toxins etc. lead to cell death and tissue damage in the liver.
- Even though the liver does regenerate it cannot keep up with the apoptosis occurring

Take home message:

******Apoptosis MUST be highly regulated and maintained within a fine balance in order for life to proliferate& maintain homeostasis******

03/09/2007

Lecture #24– Apoptosis II

NOTE: This NTC is meant to be used as a study aid to supplement your own class notes. Hence, not all of the text or diagrams contained in the lecture slides will be reproduced here.
Please send any comments or questions about NTCs to either Misha or James through e-mail at **bugs_u2reps@yahoo.ca**

Dr Shore was away therefore the lecture was given by Matthew War, a graduate student in Dr Shore's lab who specializes on Apoptosis.

Last lecture introduced programmed cell death (apoptosis), the apoptotic process, and how defects in the regulators of the core death machinery contribute to diseases.
Today we will got through the pathway that drives apoptosis

C. elegans genetic system

- *C. elegans* is a small microscopic, 959 celled multicellular worm.
- *C. elegans* make exactly 1090 cells during development, 131 cells are programmed to die, giving a 959 celled nematode.
- Sydney Brenner thought this made C *elegans* a model organism for the study of apoptosis
- John Sulston mapped the exact cells that die during development.
- Robert Horvitz did genetic mutations in many genes looking for the ones essential for cell death
- Together they won the Nobel prize in 2002 for this work in C *elegans*
- Horvitz discovered 2 genes: Ced3 and Ced4 which proved to be absolutely essential for apoptosis
- He also identified Ced9 as a suppressor of apoptosis and later Egl1 as a gene that promoted apoptosis

Genes regulating cell death:

- A 'loss of function' mutation resulting in inappropriate survival of cells would be seen if the mutated gene normally contributes to the promotion of cell death (ex. Ced 3).
- 'Loss of function' mutation in Ced 3 resulted in all 131 cells surviving. 'Gain of function' mutation in Ced 3 resulted in more than 131 cells dying, (therefore, Ced 3 is promoter of cell death).
- The second gene identified is Ced4. Knock out of Ced 4 resulted in inappropriate survival of the 131 cells.
    - Therefore Ced3 and Ced4 are essential to the apoptotic process
- Ced 3 was sequenced and found to have a mammalian homologue, which is a protease called ICE. ICE in animals induced apoptosis. This suggests that the entire apoptotic pathway could be initiated by the expression of a single active protease.
    - ICE has a Cysteine in the active domain which is able to cleave protein substrates at specific locations (after aspartic acid)
    - They are called caspases because C (cysteine) ASP (aspartic acid) ASES (proteASE).
    - Ced3 from a worm can induce cell death in a mammalian cell; therefore this suggests ICE and Ced3 are conserved protein homologues.
- When Ced 4 and 3 were expressed in bacteria, Ced 4 was found to interact with Ced 3.
- Ced 4 'gain of function' mutations introduced into animals defective in Ced 3 allowed all cells to survive. Therefore, Ced 4 operates upstream of Ced 3 genetically. Ced 4 also functions through Ced 3.

- The next gene identified through mutation was Ced 9. Loss of function of Ced 9 had an opposite effect to loss of function of Ced 3 and 4. It led to massive cell death, indicating that Ced 9 is a suppressor of cell death. It was shown that Ced 9 functions upstream of Ced 4 and through Ced 4.
- This network functions in a linear fashion: Ced 9 binds and inhibits Ced 4 (Ced 9 is upstream of Ced 4). Ced 4 then functions through Ced 3 (Ced 4 is upstream of Ced 3).
    - Egl1 can bind to Ced9 thereby inhibiting the function of Ced9, causing Ced4 be free of Ced9.
    - Egl1 has many homologues in mammalian cells: BH3-only proteins, which are regulators of Bcl2, a Ced9 homologue.

Ced3

- Initially the function of Ced3 was not identified. It was only the human homologue ICE that was identified as a protease, thus it was concluded that Ced 3 functions as protease.
    - There are 13 caspases identified in mammalian cells
- ICE is a cysteine protease, i.e., active site cysteine is catalytically driving the cleavage of protein substrates, after an aspartic acid.

Caspases fall into 3 categories

- The first class includes caspases 5, 4 and 1. They are not so much involved in apoptosis as in inflammation, generating inflammatory cytokines. If these are over-expressed they will act as apoptosis inducing caspases.
- The second group includes caspases 7, 3 and 6. These are the effector caspases, responsible for cleaving the several hundred proteins that result in the apoptotic disassembly of the cell. Their optimal sequence is DEVD which is found in PARP and ICAD, therefore these caspase perform the actual cleaving process.
    - In contrast to ICE, they don't have a long pro region, but a short pro domain
- The third group includes caspases 8, 10, 2, 9. They're called the initiator caspases. They have an optimal cleave site regions or xDxV.
    - They have long pro domain.
    - They are autocatalytic
- Caspases recognize aspartate residue and cleave at the carboxy side.
- Inflammatory cytokine generating proteins (such as ICE): usually recognize recognition sequences on the protein substrate. Upstream of the cleavage site, there are four amino acid positions recognized by the protease. Different protein substrates have different recognition sequences. For example, YVAD (tyrosine, valine, alanine, and aspartate) is recognized by ICE. DEVD is recognized by caspase 3. This allows for fine tuning of the signal and higher affinity between protease and substrate.

- What happens if a pro region is removed and replaced by a protein fragment called FKBP? (FK506 Binding Protein; a natural protein recognizing a small molecule called FK506).
    - A chemist made FK1012, which is a dimer able to bind to 2 FKBPs.
    - Initiator caspase 8 was used in which the pro region is replaced by FKBP. FK1012 was added. This triggers dimerization and very efficient processing of the enzyme into the final *tetramer* holoenzyme.
- In order for the initiator proenzyme to be activated, two molecules have to be brought into very close proximity. There's enough activity associated with the initiator proenzyme that when they are brought very close together, they can process each other through autocatalytic processing.
- If we do the same experiment using mutant proenzyme in which the active site cysteine is mutated (no longer a functional protease), there is no processing.
- If you make a knock-out mouse without caspase 3, the mouse will not be viable due to lack of apoptosis

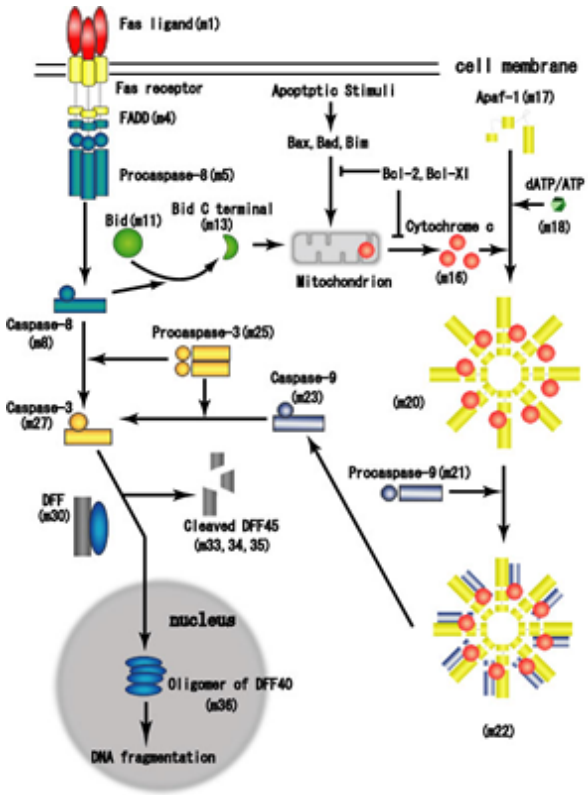
What are the signals that activate the caspase cascade??

Intrinsic and Extrinsic pathway

Extrinsic Pathway

- Focus on the death receptors that are expressed on the plasma membrane of cells
- TRAIL & FAS receptor
  - When these come in contact with their ligand this induces a conformational change on the other side of the membrane which recruits **adaptor proteins with a death domain**
    - Pro-caspase 8 has a death domain in its pro-domain
    - adaptor molecules are recruited which brings caspases into close proximity of each other and allows them to interact and autocatalytically activate
      - this causes pro-caspase 3 and 7 to become caspase 3 and 7
- the activation of pro-caspase 8 is regulated
- In the extrinsic cell pathway, the adaptor protein FADD recruits pro-caspase 8 through its death effector domain
- The activation of procaspase 8 is regulated by CFLP which can block activation. If a Dcore receptor is present in the extracellular matrix (which happens in several cancers) FAS receptor will be blocked, preventing ligand binding which inhibits intra cellular signaling and apoptosis.

Intrinsic Pathway



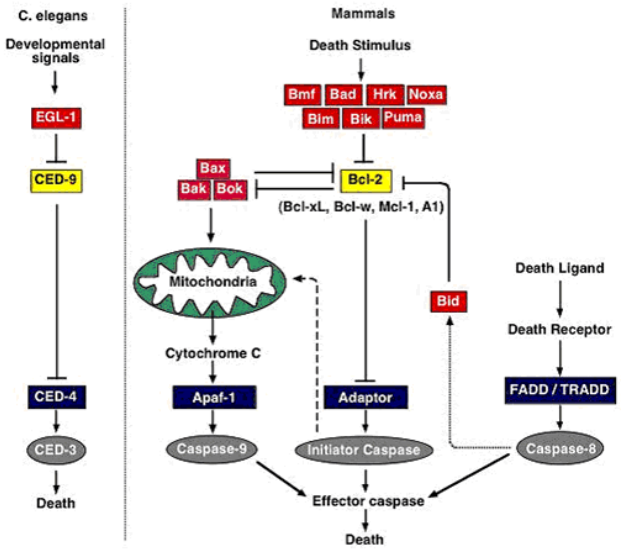
- triggered by DNA damage or oncogenes activation
- p53 can sense damage, which if not repaired, will signal apoptosis through the intrinsic pathway
- this pathway is tightly regulated through the Bcl2 family of proteins
  - it can block programmed cell death
  - it was found in a transchromosomal translocation of chromosome 14 which puts Bcl2 in range of a strong promoter which causes its normal levels to be deregulated and over-expressed
  - unlike all other oncogenes known, it did not increase cell proliferation but caused cell survival
  - it caused activation of MYC, a known oncogene, which caused cell proliferation
    - thus they act synergistically in cancers
- Other Bcl2 family members have now been identified which now include pro-apoptotic and anti-apoptotic members
  - They are characterized by their number of Bcl2 homology (BH) domains
    - Pro-survival domains include BCLXL and MCL1
    - Pro-death member domains are divided into 2 categories
      - The multi-domain effector
        - Which contain BH domains 1,2,3
      - The BH3 only domain members

- BID, BAX, and BAK
  - BH1,2,3 domain can form a hydrophobic pocket which can accommodate a BH3 domain from another member
  - BH3 only proteins can bind pro-survival BCl2, BCLXL and MCL1
  - Pro-survival Bcl2 members have a transmembrane domain on the C terminal which can target the proteins to the mitochondria
- In a healthy cell, Bcl2, BCLXL and MCL1 can bind Bax and Bak making them inactive
- when Bcl2 is in excess, it can block BAX/BAK oligomerization
- therefore, whether mitochondria responds to signal depends on ratio of Bcl-2 to BAK/BAX
- BAK/BAX are the key effectors of the majority of the apoptotic pathway
- Bcl-2 sits at the surface of the mitochondria and determines if cytochrome c can be released

BAK/BAX is the key effector of cytochrome c release – but it has to be activated by upstream signals

- when activated, they form oligomers; which activates the release of cytochrome c
- it is believed that BAK/BAX form a protein-conducting outer membrane pore that allows free diffusion of inter-membrane space proteins out of the mitochondria
- cytochrome c binds to Apaf-1 and requires ATP to force a conformational change that activates oligomerisation and recruits procaspase-9 through the CAR domain.
  - The apoptosome is a heptomeric protein that contains Apaf1, Cytochrome C and procaspase9
- Apaf-1 acts as adaptor
- Pro-caspase 9 can now catalyze effector caspases
- the release of cytochrome c from mitochondria is the key that initiates the pathway

Remember in C elegans



- Egl1 --| ced-9 --| ced4 → ced-3
  - Egl1 blocks Ced9, which blocks Ced4 which binds to Ced3 and activates apoptosis
- In Humans cells
  - Egl1 has homology to the BH3 only proteins which are upstream of Bcl2
  - Ced9 has homology to Bcl2 / Mcl1 / BCLXL
  - Ced4 has homology to Apaf1
  - Ced3 has homology to caspase 3 and 7
  - The mammalian pathway is conserved to the C *elegans* pathway
  - In a healthy cell, Bcl2, BCLXL and MCL1 can bind BAX and BAK making them inactive therefore there is a balance between pro-death and pro-survival mechanisms
  - During apoptosis there is a tip in the balance towards pro-death meaning more pro-death proteins compared to pro-survival members
    - This involves the oligomerisation of BAX and BAK and the release of cytochrome C and activation of procaspase9 which causes apoptosis
    - Both genes are knocked out in knock-out mice – these cells never respond to cell death signals
- If the signal happens to be an inducer of an oncogene, it induces BH3, activates BAK/BAX and causes cell death to remove the threat
- Double knock-out of BAK/BAX results in high cancer rate (and cell survival)
- Bcl-2 is strongly up regulated in cancer cells, so the oncogene is able to through self-proliferation

Therapeutics:

- mimic BH3 domain that fits into Bcl-2 to prevent it from interacting with BAK/BAX (a small molecule that can fit in groove to titrate Bcl-2 out)
- first genetic event is the activation of transforming oncogenes
- then need a 2nd-hit genetic event, which causes increase in Bcl-2
- normal cells won't deliver the inherent stress signal that causes BAK/BAX to oligomerize; only oncogenes do.
- therefore cancer cells can be targeted and killed by this treatment

03/14/2007

Lecture #25– Protein Targeting

NOTE: This NTC is meant to be used as a study aid to supplement your own class notes. Hence, not all of the text or diagrams contained in the lecture slides will be reproduced here.
Please send any comments or questions about NTCs to either Misha or James through e-mail at **bugs_u2reps@yahoo.ca**
Today: Apply the principles of protein targeting to the ER and mitochondria and describe how these organelles deal with these steps mechanistically.

Protein Translocation into the Endoplasmic Reticulum (ER)

- The ER
  - Is the beginning of secretory pathway.
  - Synthesizes all proteins that stay in ER or are transported to other organelles.
  - is dissected using biochemical reconstitution and genetic dissection
    - Biochemical methods (including reconstitution of the translocation pathway) are most fruitful for identification of ER mechanisms.

We will focus on the starting point beginning with how proteins get from the cytoplasm to the lumen of the ER.

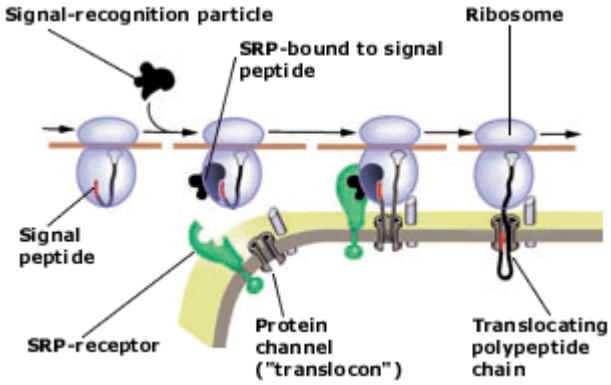
1. Targeting information resides in topogenic sequences.
   - The signal sequences that target proteins to the ER are similar but not identical.
     - Generally, these are 20 to 30 amino acids (AAs) long with a hydrophobic core of AAs which are typically preceded by positively charged AAs such as lysine or arginine.
     - Most of these signals have a cleavage site so the signal sequence is removed in the ER lumen.

Two tricks used to dissect ER translocation (*in vitro*).

   - Synchronized protein translation.
     - Take naked mRNA and add to *in vitro* protein translation system.
     - After a few minutes, add inhibitor of protein translation initiation, such as 7-methyl guanosine.
     - In this time frame, a single ribosome has had enough time to load onto the 5'end of the mRNA.
     - This method ensures that all polypeptides within a given population of mRNA are translated synchronously.
   - Truncated mRNA – another approach is a variation on the first method
     - Use in combination with the synchronized protein translation so that homogenous population of truncated proteins is generated.
     - When a ribosome reaches the mRNA termination codon, it disassembles from the chain, but if the mRNA is truncated prior to the termination codon, ribosome stops but does not dissociate.
     - Depending on the location of termination, can have the ribosome stop at any length to generate a population of truncated polypeptides.
     - These truncated proteins are then added to the ER translocation system to see what happens.
     - In addition, chemical functionalities (such as chemical cross-linking and fluorescent tags) can be incorporated into the backbone of truncated proteins in order to probe their environment during translocation.
     - The default pathway of the ER is to translocate the proteins out of the cell, there must be additional sorting signals to transport proteins downstream of the ER

2. Targeted proteins must be in a conformation competent for membrane translocation.
   - In the ER, as opposed to the other four competent organelles, translocation is co-translational

- The key players in establishing the co-translational translocation (ribosome binding) on the ER membrane were devised through protein analysis of cytosolic and surface bound proteins
- The first and most important factor that initiates this process was purified from the cytosol since translation without cytosolic proteins in the presence of the ER were not competent for translocation of the nascent chain into the organelle
  - This protein was signal recognition particle (SRP).
  - SRP is a complex of six proteins and one RNA molecule (an abundant cytoplasmic RNA called 7SL RNA) – SRP is a ribonuclear protein particle.



- Translocation competence has been dissected using synchronized protein translation.
  - Allow synchronous translation to proceed and add ER vesicles at different times after initiation of translation.
  - If the vesicles are added early on, all proteins are targeted to the lumen of ER vesicles.
  - However, if ER vesicles are added after translation is allowed to go beyond the first 70 AAs, then the vast majority of proteins are not targeted to the ER (proteins become translocation incompetent).
  - Therefore, there must be a mechanism that keeps the first 70 AAs translocation-competent.
  - SRP has a variety of functions that were dissected by removing one of the six SRP proteins at a time, placing the SRP into a translation-translocation system and observing which function was missing.

- Each SRP function is conferred by specific proteins within the SRP.
  1. SRP is involved in quality control of co-translation translocation.
     - It doesn't acts as a translational inhibitor in the absence of membranes
  2. SRP physically interacts, with p54, with the signal sequence as it emerges from the ribosome during protein synthesis.
  3. SRP acts as an intermediary between the signal sequence and the SRP receptor (SRPR)
     - The SRPR doesn't recognize the signal sequence but the SRP
- SRP is essential to maintain translocation competence

Cytosolic movement to membranes is driven by diffusion.

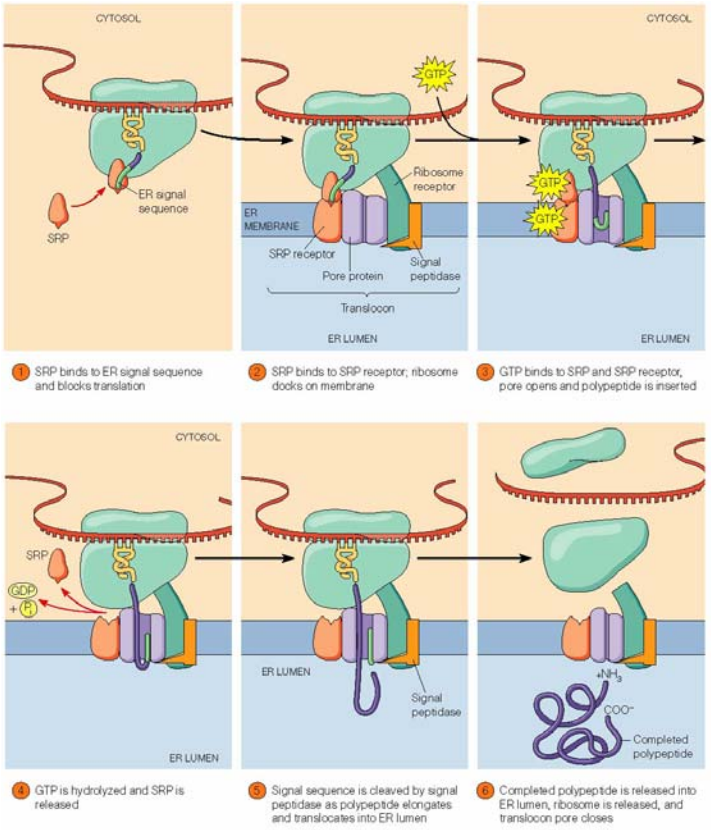
- The ribosome-nascent mRNA complex moves by diffusion until it reaches the pore complex.
  - Typically at a steady state, ribosomes that are synthesizing secretory proteins reside at the ER membrane because every time a polypeptide is released by one ribosome, another ribosome is moving down the 5'end of the mRNA

Correct membrane recognition is determined by receptors.

- When the cytosolic domain of SRPR was isolated and added to *in vitro* translation systems, it relieved translation arrest conferred by SRP.
- This receptor was discovered because a laboratory was analyzing proteins on the surface of the ER by using proteases in the hopes of isolating a fragment and analyzing it biochemically.
- They identified a protein whose cleavage site by a specific protease was near the membrane and liberated a 60kDa fragment from a 68kDA protein
- In analyzing its function, it was asked whether it could overcome inhibition by SRP
  - this was done in the absence of vesicles

    - This protein could inhibit the inhibition of SRP
  - It avidly binds SRP and therefore was named the SRP-receptor
  - It is comprised of 2proteins Alpha and Beta

- binding assays with truncations were constructed to analyze the function and binding affinity of the proteins
- In order for the nascent polypeptide to be released by the SRP and enter the pore, the high affinity interaction between SRP and SRPR must be converted into a low affinity interaction – this involves classic signal transduction.



- Nucleotides as signaling molecules are used to convert receptor recognition from high to low affinity interactions.
- Two molecules of GTP are added.
- this causes the proteins to change their conformation
- A complex of SRP, the receptor, the signal sequence and the ribosome triggers the binding of GTP to the complex
- GTP has binding sites on both p54-SRP and Alpha-SRPR.
- The first GTP molecule binds the alpha-receptor, disengaging the receptor from the SRP, presumably due to conformational changes induced in the receptor.
- The second molecule of GTP binds the SRP complex and is hydrolyzed to GDP, enabling the SRP to disengage from the signal sequence.
- Now, the signal sequence is very close to translocation machinery and can now interact with the translocation sites.

Protein insertion into or translocation across membranes is (usually) mediated by pore complexes.

  - The aqueous Sec61 pore complex consists of three proteins, Sec61 α, β and γ.
    - It was first identified in yeast as a secretory protein
    - These are present in multiple copies that form a large pore 20 to 60 Å in diameter, which is large enough to accommodate lots of protein.
    - These pore complexes are large enough to freely transport a lot of metabolites and even small proteins by diffusion – one condition for protein translocation is that these pores are regulated to prevent diffusion of metabolites.
  - Pore complexes are gated – they are open when protein is to be translocated and closed when the polypeptide is not in transit triggered by the signal sequence itself.
    - The ribosome often acts as a gate itself.
    - The ribosome engages the pore complex and sits on top of it, and blocks metabolites from diffusing into or out of the ER lumen.

Unidirectional protein translocation is an energy consuming process.

  - ATP is the major energy currency and is coupled to protein co-translational translocation.

        - Basically, ATP is already required for protein translation and since the ER involves co-translation, the newly synthesized protein has nowhere else to go but through the pore complex.
    - Chaperones in the ER lumen are ATP consumers.
        - These capture the translocated proteins and help them fold into their final conformation, which exists at a lower free energy state compared to the nascent polypeptide chain at the other side of the ER membrane.
        - This process establishes favourable thermodynamics for unidirectional translocation into the lumen.

Translocating polypeptides fold on trans side of membrane.

- Aided by chaperones.
- signal sequence is removed on trans side of membrane

Protein insertion into membranes is coupled to translocation across membrane.

- Membrane proteins contain membrane anchor hydrophobic sequences. The pore opens laterally and the chain is inserted into the lipid bilayer.

Orientation of the translocated protein depends on the location of the N-terminus of the protein

Following translocation, proteins are sorted.

- Topogenic sorting signals:
    - Default pathway (without any sorting signal) is the outside of the cell.
    - For proteins resident in the ER lumen, typically have at their C-terminus a tetrapeptide sequence KDEL (lysine-aspartate-glutamate-leucine), which serves as a retention signal.
    - Mannose-6-phosphate sugar is added to proteins that are destined for the lysosome.

Principles and Steps in Protein Targeting to the Mitochondrion

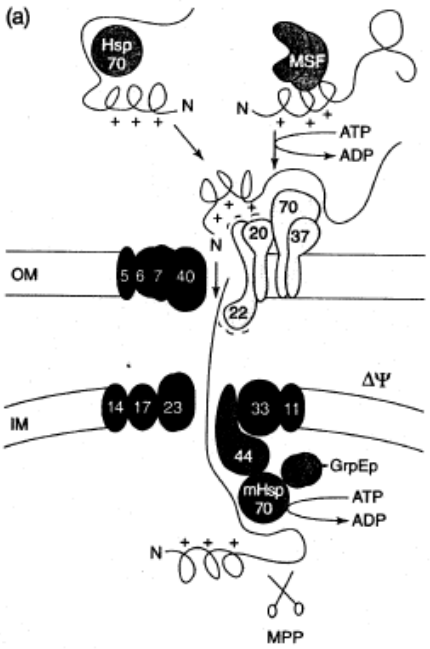
- This is a very different system compared to the ER with a different set of machinery.
- Protein targeting is post-translational as opposed to co-translational; however the underlying principles are similar.
- The techniques used to dissect the mitochondrial translocation system are different from those used on the ER, but with the same underlying principle.
    - One trick to analyze this system was developing translocation intermediates with precisely controlled lengths so that these proteins would stop and accumulate at different steps to be analyzed for their protein-protein interactions.
    - In the case of mitochondria, proteins are translocated post-translationally; therefore protein truncation was performed differently compared to the ER.
        - Proteins targeted to the mitochondrion were fused at their N-terminus to a cytoplasmic protein, the DHFR (dihydrofolate reductase) enzyme
        - DHFR is a small monomer with a potent inhibitor methotrexate (MTX) that binds the active site of DHFR with extremely high affinity
        - DHFR was fused to the signal sequence after which MTX was added, locking DHFR into a globular conformation that cannot be unfolded.
        - One principle for translocation into the mitochondrion is that proteins must be unfolded to be competent for translocation.
        - Depending on where the DHFR was fused, it will arrest the translocation complex at different points.
        - The first experiments asked how much mitochondrial destined protein has to be on DHFR until you see cleavage of the signal sequence.
            - The answers was short i.e.: 50 Amino-acids
            - The only way to resolve this was to deduce that translocation occurs simultaneously through both the outer and inner membrane

- The default pathway of translocation into the mitochondria is into the matrix

Targeted proteins must be in a conformation competent for membrane translocation.

- Translocation competence is maintained by specific chaperones that interact with newly formed proteins, presumably to keep the signal sequence freely available for recognition by the mitochondrion.

Cytosolic movement to membranes is driven by diffusion.



Correct membrane recognition is determined by receptors

- Unlike the ER, there is direct recognition between the signal sequence and receptor.
  - Mitochondrion receptors are comprised of multiple proteins.
  - TOM (translocase of the outer membrane) and TIM (translocase of the inner membrane) – these names are followed by a number representing size of the protein (i.e. TOM 20 is a 20 kDa protein and part of the receptor at the outer membrane).
  - TOM70 and TOM20 are the major outer membrane translocases
- It is not yet understood how the tight binding between the pore complexes (predominately made of TOM40) is derived.

Unidirectional protein translocation is an energy consuming process.

- The pores contain an aqueous environment, providing favourable thermodynamics.
- The energetics of this system are complex.
- The signal sequence is positively charge. The mitochondrion has its own energy currency, the electrochemical gradient across the inner membrane, which is net negative on the inside and net positive on the outside.
  - It is believed that this electrochemical potential across the inner membrane allows the sequence peptide to electrophorese across TIM complexes into the matrix.
- The second energy component to be coupled to translocation is ATP hydrolysis.
  - This is coupled to translocation by mHSP70 (the "m" stands for mitochondrial), a chaperone that is associated with a translocated protein on the inner membrane.
  - Through ATP hydrolysis, HSP70 continually binds and releases polypeptide, pulling it through the pore complex
  - mHSP70 also aids proper folding so the proteins achieves the most thermodynamically favourable conformation
  - Signal sequence is typically cleaved as it emerges into matrix.

Following translocation, protein is sorted to final destination.

- Topogenic sorting signals:
  - Default pathway in absence of any other sorting is the matrix compartment.
  - The only other compartment is the space between the inner and outer membrane, but how proteins are sorted to this location is still debated.
    - It may first go to the matrix and then back to the inter membrane space or simply move laterally through the translocation pore
  - proteins with membrane anchors are released during translocation into surrounding lipid bilayer
    - the context of a 20AA hydrophobic sequence triggers release into IMM or OMM

In context to exams, we are stressing learning the principles of the machinery and not, for example, the kDa weights of the proteins.

03/16/2007

Lecture #26– Protein Turnover

NOTE: This NTC is meant to be used as a study aid to supplement your own class notes. Hence, not all of the text or diagrams contained in the lecture slides will be reproduced here.
Please send any comments or questions about NTCs to either Misha or James through e-mail at **bugs_u2reps@yahoo.ca**
Today's lecture is going to focus on protein degradation and a lot of the processes that were seen in protein sorting are going to be applied to degradation.

The amount of any given protein in a cell at steady state is dependent on its rate of synthesis and degradation. When rate of synthesis equals rate of degradation, the protein is at a specific level, which can be changed by affecting its synthesis by transcription and translation or by affecting degradation.
The degradation rate of any given protein is designated by its $T_{1/2}$ or half-life, described as the time it takes to deplete the concentration of a given protein in half for a given time characteristic on the protein in question.

The advantage of a protein that is controlled by its degradation amount and not by the regulation on its translation or transcription is that a response is immediate as opposed to regulating its creation which could take time to show any appreciable difference.
We are mainly speaking of proteins that have a role in the cell cycle, proliferation, cell death, etc.

Protein Degradation falls into 3 categories

1) Steady State Degradation
    - When proteins are continuously being degraded
2) Elimination as Quality control
    - Degradation of misfolded, mutated, non-functional proteins whose build-up could prove harmful to the cell
    - These proteins are degraded fairly quickly in order to prevent harm to the cell
3) Degradation for Specific Regulation
    - Degradation in order to keep the concentration of a given protein at homeostatic levels

Two Major Systems for Protein Degradation:

1) Lysosomes
    - Previously discussed in ER targeting
    - A membrane organelle packed with over 50 hydrolytic enzymes responsible for degrading macromolecules.
    - Part of the steady state degradation system
    - Autophagy
        - the lysosome engulfs major components of the cytoplasm and even organelles
        - Occurs in response to stress; especially nutrient/starvation stress
            - Allows cytoplasmic components and macromolecules to be recycled back to their building blocks to allow for synthesis of new proteins
            - Certain cancers can also use autophagy to replenish nutrients

2) Proteasome
    - able to degrade proteins with a $t_{1/2}$ anywhere from a couple minutes to one month
    - there has to be a signaling mechanism in order to regulate the degradation by the proteasome
        - the signal that gets a protein tagged for the proteasome is a small protein called Ubiquitin

Ubiquitin

- The pathway which is involved in cycle regulation, cell death or signal transduction is an ATP dependent pathway and depends on the protein receiving an ubiquitin signal.
- It is a small protein; 76 amino acids in length (8 kDa)
- It is very conserved; only three amino acid difference between yeast and human ubiquitin
- Represents a signal for delivering protein to protein degradation machinery
- It has a glycine at the C terminus
- The carboxylic acid at the C terminus which can make a covalent peptide bond with the ε amino group of lysine of a protein target.
- The transfer of ubiquitin onto the side chain of lysine in a protein is a targeting signal for its degradation
- There are many enzymes involved in the transfer of ubiquitin to proteins since degradation through this pathway is highly involved in cellular regulation

The enzymes that transfer ubiquitin to the lysine side chain fall into three classes:

There are multiple members in each category with multiple substrates

1. E1 enzymes: ubiquitin activating enzymes
2. E2 enzymes: ubiquitin conjugating enzymes
3. E3 enzymes: ubiquitin protein ligases

Regulation of E3 for example will regulate the proteins ubiquitinated by E3 only.

Steps in Ubiquitination

1) Activation of ubiquitin (Ub) by E1 in two steps involves ATP
    - The reaction is a simple nucleophilic substitution reaction
    - It uses the energy of hydrolysis of ATP to generate Ub conjugated to E1
    - First, an ubiquitin-AMP intermediate is formed by the attack of the C-terminus carbonyl oxygen of ubiquitin on the phosphate in ATP. Pyrophosphate is released.
    - Then, the thiol group of E1 attacks the carbonyl carbon of Ub and AMP is released as the leaving group. Ub is now covalently attached by a thioester linkage to E1
    - Ub is now reactive enough to be transferred to E2
2) The E2 enzyme, by another nucleophilic substitution, takes the ubiquitin away from E1 and is now conjugated to E2.
    - There are just a few E1 enzymes responsible for activating, while there are many more types of E2 enzymes, which can ultimately determine which protein is targeted.
3) Ubiquitin is then transferred to the protein while E2 is left behind.
    - Catalyzed by E3 ligase, which recognizes the activated E2 and the targeted protein
    - A complex is formed between E3 ligase, E2 and the protein.
    - E3 ligase is responsible for transferring Ub to the protein target, now tagged for degradation
    - Ub is attached to the protein by an amide bond to lysine, which is internal in the protein. This is an isopeptide (amide) bond.
    - Ubiquitin represents a signal for delivery of protein to the proteasome

Rate of Protein Degradation

- Rate of degradation or half life is determined by the rate of polyubiquitination. Once a protein is polyubiquitinated, it is committed to rapid degradation by the proteasome.
- Degradation signals can be intrinsic with the primary sequence of proteins. There are degradation signals that occur at the N-terminus of proteins.
    - N end rule – it is the amino acid at the N terminus which determines the rate of polyubiquitination

- If methionine is found at the N-terminus then $t_{1/2}$ is usually > 20 hours
- If arginine is found at the N-terminus then $t_{1/2}$ is usually < 2 minutes
- So there's great variation in the half life depending on the amino acids at the N-terminus
- Note: these signals within the polypeptide determine the rate of degradation assuming the degradation machinery is fully active

If one manipulates the machinery, then one can convert a protein that has a short half life into one that has a longer half life without affecting the synthesis of the protein

An example of this mechanism is the regulation of p53:

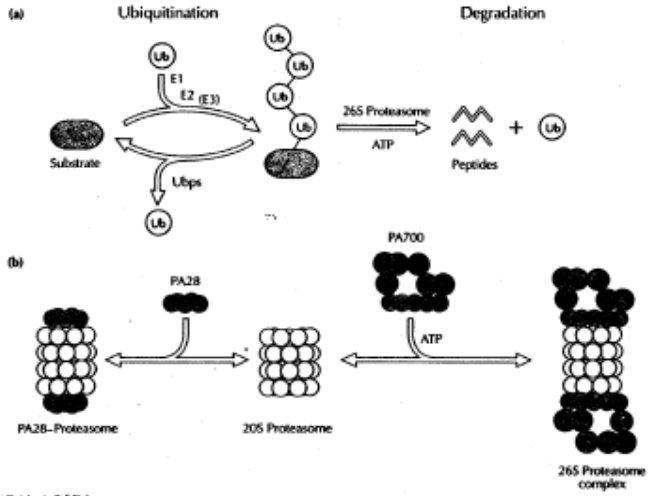
- P53 has multiple binding partners (over 75)
- It is characterized as the "guardian of the genome"
- p53 integrates many signals and regulate cell cycle and cell death
- It is regulated through degradation
- It is present in cell at very low levels because it has an N-terminal amino acid that dictates it rapid turn over →is quickly degraded
- It has to respond quickly to signaling of cell damage
- Mdm2, a major E3 ligase, is responsible for polyubiquitination of p53
- The E3 ligase couples p53 to signaling pathways
  - If the cell is under DNA damage stress, this can induce a signaling pathway that results in regulation of E3 ligase though phosphorylation, which inactivates it.
  - Therefore, newly synthesized p53 is not being degraded, so its level in the cell increases. It turns on genes involved in DNA repair and cell cycle arrest
  - This quality control mechanism allows cells to recover from DNA damage

Mcl-1:

- This protein helps to keep cells alive; high levels prevents the cell from undergoing apoptosis
- It is a pro-survival Bcl2 family protein
- Like p53, it rapidly turns-over
- When a cell receives damaging signals that corrupt it, an E3 ligase is activated that can target Mcl-1 and send it to the proteasome, allowing levels of Mcl-1 to go down. This leaves the cell unprotected and the cell can undergo apoptosis.

Targeting & the Proteosome

- A minimum of 4 Ub must be attached to tag a protein to the proteasome for degradation
- Ubiquitin has multiple lysine residues in it. You can therefore get isopeptide bonds between the C terminus of ubiquitin and lysine side chains of another ubiquitin.
- Therefore it can build chains of ubiquitin, where the C terminus of one Ub is ligated through an isopeptide bond to the lysine of an ubiquitin further downstream forming a polyUb chain which becomes a signal for targeting to the 26S proteasome.



- The polyubiquitinated protein reaches the proteasome by diffusion and is recognized by the 26S
  - 26S has a size of 2 million Da with many subunits.
- The proteasome is a massive protein complex found in the cytoplasm and the nucleus

- It is comprised of a core, the 20S proteasome, which is made up of 4 rings of 7 protein subunits (28subunits in total) which have different functions
- Its function was worked out in a rabbit reticulocyte lysate (RRL).
- Many of the subunits in the 20S proteasome have protease activity
- The active sites of the protease are found in a core of the proteasome
    - they all face the interior of the proteasome
- There must be a recognition mechanism on the proteasome that recognizes polyubiquinated proteins
- The 20S proteasome, in addition to the core, is also associated with a variety of subunits on the surface of the core; one is a 700 kDa "recognition site".
    - They recognize ubiquitinated proteins and can deliver them to the core.
- The protein is then unfolded, which is an ATP dependent reaction
- It's unfolded so it can be translocated down into the proteasome core where it can be attacked by the proteolytic active sites on the surface of the subunits of the core 20S component

How do proteins from the ER get to the proteasome for degradation?

- Proteasomes are in the cytoplasm and the nucleus.
- Nothing is known about how mitochondrial proteins are brought to the proteasome for degradation
- 20% of proteins delivered in the ER are degraded
- The pathway for the extrusion of proteins from the ER (retro-translocation) for degradation was determined using inhibitors of the proteasome
    - Certain ER proteins increased in level, so they are likely degraded by the proteasome
    - So there must be a pathway to transport misfolded proteins from the ER to the cytoplasm
- If a protein remains misfolded for a certain amount of time in the ER it is retro-translocated out of the ER to be degraded

Protein Trafficking Diseases

- Genetic diseases associated with the malfolding of proteins in the ER
- The most studied one is cystic fibrosis (CF)
- In most cases, patients have a single amino acid defect in a protein called cystic fibrosis transmembrane regulator (CFTR)
- The reason for this accumulation is because CFTR is a chloride pump regulator, and there's a metabolite imbalance which leads to the buildup of fluid and mucus in the lungs
- 70% of CF cases are caused by the Δ508 phenylalanine point mutation
- The missing phenylalanine does not affect the function of CFTR, but induces mal-folding of the protein.
- Thus when it is delivered into the lumen of the ER, it becomes a substrate for retro-translocation back into the cytosol and targeted to the proteasome. Thus not enough CFTR gets to the plasma membrane and chloride pumping is compromised.

- The sec61 translocon pore is the how misfolded proteins leave the ER
    - sec61 machinery can deliver proteins into and out of the ER
- It is the unfolding of the protein, through recognition by chaperones that deliver these proteins back to the sec61 translocon through a signal recognition mechanism.

- We could conduct an experiment in which we knock-out the chaperones that aid in the folding of the CFTR protein
- This would give the protein a larger time frame to properly fold, and it could hold an unfolded status for a longer time
- If we increase the time frame we could get the mutant protein into the ER and it would actually be functional and pump chloride

- The proteasome is actually a good target for cancer therapy
- There is a drug called Velcade (bortezomib) which acts to inactivate proteases within the proteasome which inhibits protein degradation.
- Cancer cells are rapidly cycling and involved in increased signal transduction. By adding a proteasome inhibitor, the signal transduction will be affected causing an increase of proteins that should not be elevated otherwise.
- Some of these proteins are toxic to the cell and thus the cancer cell dies
- Velcade also deranges pro-life proteins (ie Mcl1) which cause the block of induction of apoptosis and therefore it could cause an opposite effect to what is wanted.
- A drug that inhibits proteasomes but does not affect Mcl1 would be far more effective.