Cytoskeleton :

Structure and Movement

Using this book: This book is designed to be used in both introductory and advanced cell biology courses. The primary text is generally on the left side of the vertical divider, and printed in black. Details that are usually left to an advanced course are printed in blue and found on the right side of the divider. Finally, additional biomedically relevant information can be found in red print on either side of the divider.

When a eukaryotic cell is taken out of its physiological context and placed in a plastic or glass Petri dish, it is generally seen to flatten out to some extent. On a precipice, it would behave like a Salvador Dali watch, oozing over the edge. The immediate assumption, particularly in light of the fact that the cell is known to be mostly water by mass and volume, is that the cell is simply a bag of fluid. However, the cell actually has an intricate microstructure within it, framed internally by the components of the cytoskeleton.

As the name implies, the cytoskeleton acts much like our own skeletons in supporting the general shape of a cell. Unlike our skeletons though, the cytoskeleton is highly dynamic and internally motile, shifting and rearranging in response to the needs of the cell. It also has a variety of purposes beyond simply providing the shape of the cell. Generally, these can be categorized as structural and transport. While all three major components of the cytoskeleton perform each of these functions, they do not do so equally, as their biophysical characteristics are quite different. With respect to structure, at some point in the life of every cell, it must change shape, whether simply increasing or decreasing in size, or a more drastic alteration like the super-elongated form of neurons with axons, the cytoskeleton must be able to respond by dynamically increasing and decreasing the size of the internal structures as needed. Structure also applies to the relative position of internal cellular elements, such as organelles or proteins, to one another. In many highly specialized cells, the segregation of particular structures within certain parts of the cell is crucial for it to function. Transport refers to the movement of molecules and organelles within the cell as well as movement of the cell as a whole. We just discussed intracellular movement of proteins and lipids by way of vesicles in the last chapter. Those vesicles, as we will see in this chapter, are not just floating from one place to another; they are moved purposefully and directionally along the cytoskeleton like cargo on highways or railroad tracks. With respect to whole cell movement, this can range from paddling or swimming by single-celled organisms to the stereotyped and highly coordinated crawling of many cells from their point of origin to their eventual destination during the development of a metazoan organism or the movement of fibroblasts to heal a cut in your skin.

Although the genes are not particularly well conserved, a combination of genetic similarity and protein structure have confirmed the presence of prokaryotic proteins that are related to eukaryotic cytoskeletal proteins in both form and function. Compared to the eukaryotic cytoskeleton, study of prokaryotic proteins is very recent, and for a long time, there was an assumption that prokaryotes did not have or need cytoskeletal architecture. FtsZ, the bacterial equivalent of tubulin, was discovered in 1980 but most of the work on it has occurred in the last decade. MreB is an actin-like protein, first compared to actin in 1992, and crescentin, an intermediate filament class protein, was only described in 2003. For comprehensive review of prokaryotic cytoskeleton proteins, see Graumann, P.L., *Ann. Rev. Microbiology* **61**:589-618, 2007.

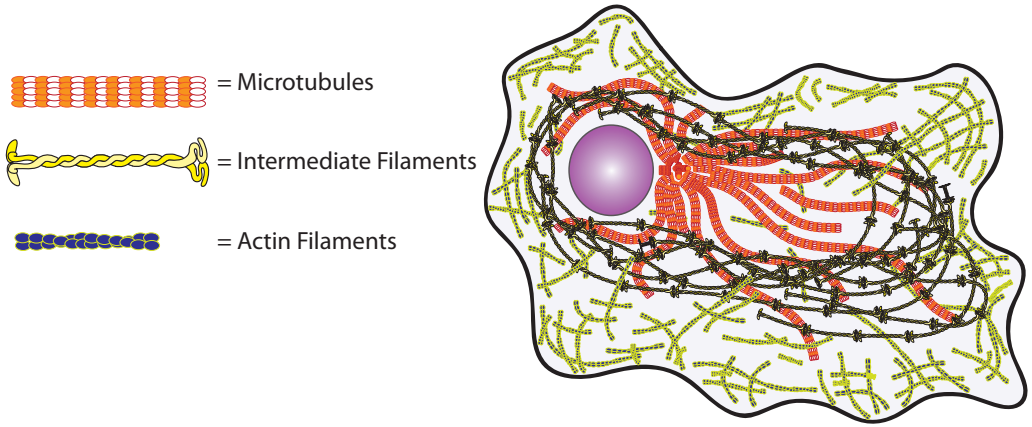


Figure 1. Cytoskeletal element distribution in a prototypical eukaryotic cell. The purple ball is the nucleus.

The three major components of the cytoskeleton are microtubules, microfilaments, and intermediate filaments. Each of these are polymers composed of repeating subunits in specific arrangements. With just a quick glance (fig. 1), it is very clear that the intermediate filaments will likely play a significantly different role from either microtubules or microfilaments. Because the IF's are made of long fibrous subunits that coil around one another to form the filament, there is clearly a great deal of contact (which facilitates formation of hydrogen bonds, aka molecular velcro^{tm}) between subunits providing great tensile strength. It is very difficult to break these subunits apart, and thus the IF's are primarily used for long-term or permanent load-bearing purposes. Looking at the other two components of the cytoskeleton, one can see that with the globular instead of fibrous shape of the subunits, the maximum area of contact between subunits is greatly limited (think of the contact area when you push two basketballs together), making it easier to separate the subunits or break the microfilament or microtubule. The cell can use this characteristic to its advantage, by utilizing these kinds of cytoskeletal fibers in dynamic situations where formation or destruction of intermediate filaments would take far too long. We now address these three groups of cytoskeletal elements in more detail.

Intermediate Filaments

"Intermediate filaments" is actually a generic name for a family of proteins (grouped into 6 classes based on sequence and biochemical structure) that serve similar functions in protecting and shaping the cell or its components. Interestingly, they can even be found inside the nucleus. The nuclear lamins, which constitute class V intermediate filaments, form a strong protective mesh attached to the inside face of the nuclear

Most intermediate filaments fall between 50-100 kDa, including keratins (40-67 kDa), lamins (60-70 kDa), and neurofilaments (62-110 kDa). Nestin (class VI), found mostly in neurons, is an exception, at approximately 240 kDa.

membrane. Neurons have neurofilaments (class IV), which help to provide structure for axons — long, thin, and delicate extensions of the cell that can potentially run meters long in large animals. Skin cells have a high concentration of keratin (class I), which not only runs through the cell, but connects almost directly to the keratin fibers of neighboring cells through a type of cellular adhesion structure called a desmosome (described in the next chapter). This allows pressure that might be able to burst a single cell to be spread out over many cells, sharing the burden, and thus protecting each member. In fact, malformations of either keratins or of the proteins forming the desmosomes can lead to conditions collectively termed *epidermolysis bullosa*, in which the skin is extraordinarily fragile, blistering and breaking down with only slight contact, compromising the patient's first line of defense against infection.

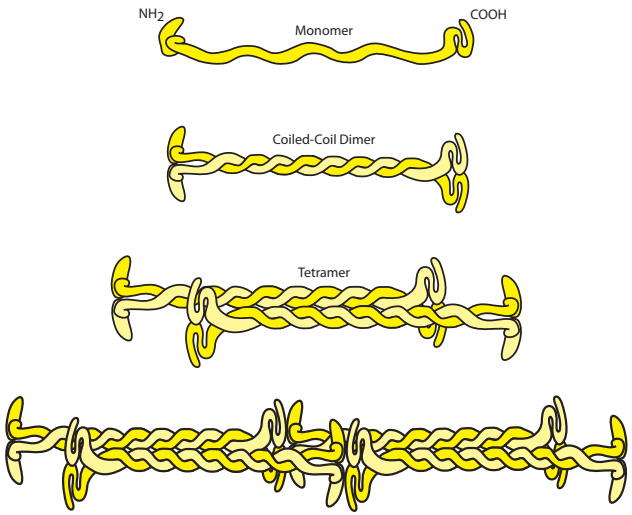


Figure 2. Intermediate filaments are composed of linear subunits that wrap around each other and interact very tightly.

Structurally, as mentioned previously, all intermediate filaments start from a fibrous subunit (fig. 2). This then coils around another filamentous subunit to form a coiled-coil dimer, or protofilament. These protofilaments then interact to form tetramers, which are considered the basic unit of intermediate filament construction. Using proteins called *plectins*, the intermediate filaments can be connected to one another to form sheets and meshes. Plectins can also connect the intermediate filaments to other parts of the cytoskeleton, while other proteins can help to attach the IF cytoskeleton to the cell membrane (e.g. desmoplakin). The most striking characteristic of intermediate filaments is their relative longevity. Once made, they change and move very slowly. They are very stable and do not break down easily. They are not usually *completely* inert, but compared to microtubules and microfilaments, they sometimes seem to be.

Epidermolysis bullosa simplex is a collection of congenital diseases caused by mutations to the keratin genes KRT5 or KRT14, or to the plectin gene PLEC1. These mutations either weaken the polymerization of keratin into filaments, or the interaction between keratin filaments. This leads to the inability of each individual cell to maintain structural integrity under pressure. Another type of EB, junctional epidermolysis bullosa (JEB), is caused by mutations to integrin receptors (b4, a6) or laminins. This includes JEB gravis or Herlitz disease, which is the most severe, often leading to early postnatal death. JEB is also related to dystrophic epidermolysis bullosa (DEB) diseases such as Cockayne-Touraine, each of which s due to a mutation in collagen type VII. The gene products involved in JEB and DEB are discussed in more detail in the next chapter. They play a role in adhering the cells to the basememnt membrane, and without them, the disorganization of the cells leads to incomplete connections between the epidermal cells, and therefore impaired pressure-sharing.

Some forms of *Charcot-Marie-Tooth disease*, the most common inherited peripheral nerve disease, are also linked to mutations of intermediate filament genes. This disease, also known as peroneal muscular atrophy or hereditary motor sensory neuropathy, is a non-lethal degenerative disease primarily affecting the nerves of the distal arms and legs. There is a broad variety of CMT types and causes, the most common being malformations of Schwann cells and the myelin sheath they form. CMT type 2 is characterized by malformations of the peripheral nerve axons, and is linked to mutations of lamin A proteins and of light neurofilaments. The causal mechanism has not yet been established; however, the neurofilaments are significant elements in maintaining the integrity of long axons.

Actin Microfilaments

Microfilaments are also known as actin filaments, filamentous actin, and f-actin, and they are the cytoskeletal opposites of the intermediate filaments. These strands are made up of small globular actin (g-actin) subunits that stack on one another with relatively small points of contact. You might envision two tennis balls, one fuzzy and the other covered in velcro hooks. Even if you push hard to mush them together, the area of contact between the balls (i.e. the area available for H-bonding between subunits) is fairly small compared to the overall surface area, or to the area of contact between IF subunits. They will hold together, but they can also fall apart with relatively little force. Contrast this with intermediate filaments, which might be represented as two ribbons of velcro hooks or loops. Considerably more work is required to take them apart. Because there are fewer H-bonds to break, the microfilaments can be deconstructed very quickly, making it suitable for highly dynamic applications.

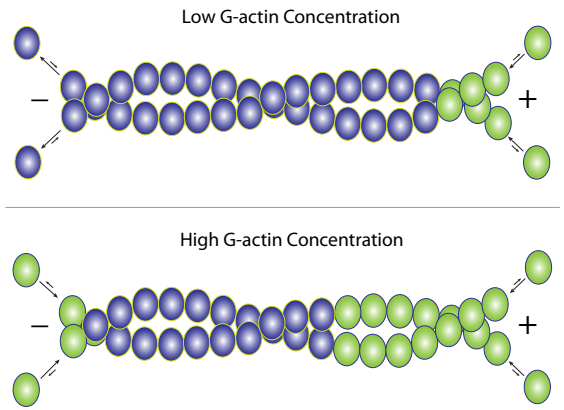


Figure 3. Actin microfilaments have a (+) and (-) end. When the free (globular) actin concentration is low, actin is primarily added to the (+) end, and lost from the (-) end. However at high levels of g-actin, new monomers can potentially add onto the filament from either end.

When the actin subunits come together to form microfilaments, they interact directionally. That is, subunits have a "top" and a "bottom", and the top of one subunit always interacts with the bottom of another. If we go to the "bottom"-most subunit of a filament, the open end is called the minus (-) end, while the opposite end, which incidentally sees more additive action, is called the plus (+) end. Microfilaments are also said to have polarity, but again this is only in the sense of having directionality, and has nothing to do with electrical charge. Individual microfilaments can exist, but most microfilaments in vivo are twisted pairs. Unlike DNA; however, microfilament pairs are not antiparallel: both strands have the same directionality.

The formation of filaments from g-actin is an ATP-dependent process, although not in the conventional sense of utilizing the energy released in hydrolysis. Instead, the globular actin subunits will only bind with another g-actin subunit if it has first bound an ATP. If the g-actin has bound ADP, then it must first exchange the ADP for ATP before it can be added onto a filament. This alters the conformation of the subunit to allow for a higher-affinity interaction. A short time later, hydrolysis of the ATP to ADP (with release of P_i) weakens the affinity but does not directly cause dissolution of the subunit binding. The hydrolysis is brought about by the actin itself, which has this ATPase enzymatic activity built in.

Although f-actin primarily exists as a pair of filaments twisted around each other, addition of new actin occurs by the addition of *individual* g-actin monomers to each filament (fig. 3). Accessory proteins can be used to help or hinder either the building or breakdown of the filaments, but the primary mechanism is essentially self-regulating. When free g-actin levels are high, elongation of actin filaments is favored, and when the g-actin concentration falls, depolymerization of f-actin predominates. Under average physiological conditions, though, what is often seen in actin microfilaments is an effect called treadmilling. Since actin is mostly added onto one end but removed from the other, the net effect is that any given actin monomer in a filament is effectively moving from (+) end to (-) end even if the apparent length of the filament does not change.

In most cell types, the greatest concentration of actin-based cytoskeletal structures is found in the periphery of the cell rather than towards the center. This fits well with the tendency of the edges of the cell to be more dynamic, constantly adjusting to sense and react to its environment. Clearly, the polymerization and depolymerization of actin filaments is much faster than for intermediate filaments. The big exception to the actin-in-periphery rule is found in muscle cells. Actin filaments, and the myosin motor proteins that work on them, are the basis for muscle cell contraction, and fill up most of the muscle cells, not just the periphery. We will discuss the role of actin in both types of cell movement later in the chapter.

Microtubules

Microtubules are made up of two equally distributed, structurally similar, globular subunits: α and β tubulin. Like microfilaments, microtubules are also dependent on a nucleotide triphosphate for polymerization, but in this case, it is GTP. Another similarity is that microtubules have a polarity in which the (-) end is far less active than the (+) end. However, unlike the twisted-pair microfilaments, the microtubules are mostly

Microtubule stability is temperature-dependent: if cooled to 4°C, microtubules fall apart into $\alpha\beta$-tubulin heterodimers. Warmed back up to 37°C, the tubulin repolymerizes if there is GTP available.

found as large 13-stranded (each strand is called a protofilament) hollow tube structures. Also, the α and β tubulin used for building the microtubules not only alternate, but they are actually added in pairs. Both the α-tubulin and β-tubulin must bind to GTP to associate, but once bound, the GTP bound to α-tubulin does not move. On the other hand, GTP bound in the β-tubulin may be hydrolyzed to GDP. GDP-bound αβ-dimers will not be added to a microtubule, so similar to the situation with ATP and g-actin, if the tubulin has GDP bound to it, it must first exchange it for a GTP before it can be polymerized. Although the affinity of tubulin for GTP is higher than the affinity for GDP, this process is usually facilitated by a GEF, or guanine nucleotide exchange factor. As the signal transduction chapter will show in more detail, this type of nucleotide exchange is a common mechanism for activation of various biochemical pathways.

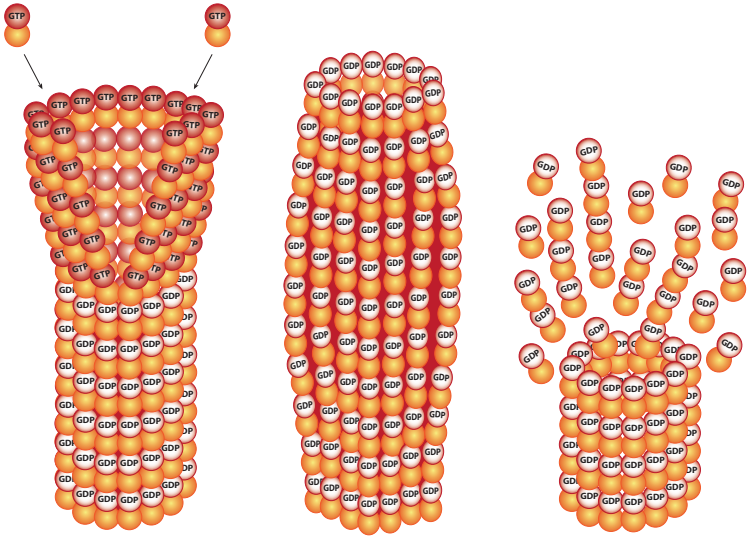


Figure 4. Microtubules exhibit dynamic instability. GTP-bound αβ-tubulin dimers are added onto the microtubule. Once the GTP is hydrolyzed, the conformational shift strains the microtubule, which will tend to break apart unless new tubulin dimers are added to stabilize the structure.

Again like actin, the tubulin itself has enzymatic activity, and over time, the GTPase activity hydrolyzes the GTP to GDP and phosphate. This changes the attachment between β-tubulin of one dimer and the α-tubulin of the dimer it is stacked on because the shape of the subunit changes. Even though it isn't directly loosening its hold on the neighboring tubulin, the shape change causes increased stress as that part of the microtubule tries to push outward. This is the basis of a property of microtubules known as *dynamic instability*. If there is nothing to stabilize the microtubule, large portions of it will fall apart. However, as long as new tubulin (which will have GTP bound) is being added at a high enough rate to keep a section of low-stress "stable"-conformation microtubule (called the GTP cap) on top of the older GDP-containing part, then it stabilizes the overall microtubule. When new tubulin addition slows down, and there is only a very small or nonexistent cap, then the microtubule undergoes a *catastrophe*

in which large portions rapidly break apart. Note that this is a very different process than breakdown by depolymerization, which is the gradual loss of only a few subunits at a time from an end of the microtubule. Depolymerization also occurs, and like with actin, is determined partially by the relative concentrations of free tubulin and microtubules.

From a physical standpoint, the microtubule is fairly strong, but not very flexible. A microfilament will flex and bend when a deforming force is applied (imagine the filament anchored at the bottom end standing straight up, and something pushing the tip to one side). The microtubule in the same situation will bend only slightly, but break apart if the deforming force is sufficient. There is, of course, a limit to the flexibility of the microfilament and eventually, it will also break. Intermediate filaments are slightly less flexible than the microfilaments, but can resist far more force that either microfilaments or microtubules.

Microtubule Organizing Centers

Microtubules, like microfilaments, are dynamic structures, changing in length and interactions to react to intra- and extra-cellular changes. However, the general placement of microtubules within the cell is significantly different from microfilaments, although there is some overlap as well as interaction. Microfilaments do not have any kind of global organization with respect to their polarity. They start and end in many areas of the cell. On the other hand, almost all microtubules have their (-) end in a perinuclear area known as the MTOC, or microtubule organizing center and they radiate outward from that center. Since the microtubules all radiate outward from the MTOC, it is not surprising that they are concentrated more centrally in the cell than the microfilaments which, as mentioned above, are more abundant around the periphery of the cell. In some cell types (primarily animal), the MTOC contains a structure known as the *centrosome*. This consists of a centriole (two short barrel-shaped microtubule-based structures positioned perpendicular to each other) and a poorly defined concentration of pericentriolar material (PCM). The centriole is composed of nine fibrils, all connected to form a cylinder, and each also connected by radial spokes to a central axis. The electron micrograph in figure 5 shows a cross-section of a centriole. In it, each fibril is shown to actually be a fused triplet of microtubules.

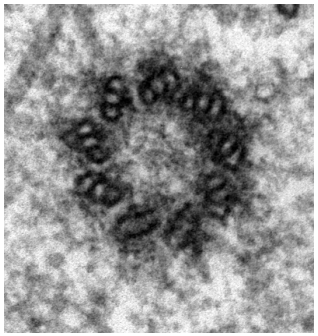


Figure 5. An electron micrograph depicting the cross-section of a centriole in an embryonic mouse brain cell. L. Howard and M. Marin-Padilla, 1985

Inhibition of γ-tubulin function by antibody blocking, RNA interference of expression, and gene knockout confirm that without γ-tubulin function, the microtubule structures did not form. In addition, it appears to be play roles in coordination of late mitosis (anaphase onwards).

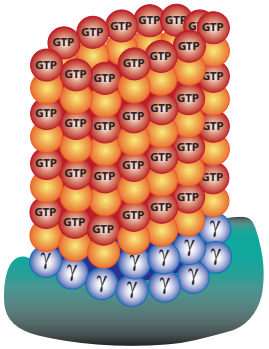


Figure 6. γ-tubulin ring complex facilitates microtubule nucleation.

However, in each triplet, only one is a complete microtubule (designated the A tubule), while the B and C tubules do not form complete tubes (they share a wall with the A and B tubules, respectively). Interestingly, the centrioles do not appear to be connected to the cellular microtubule network. However, whether there is a defined centrosome or not, the MTOC region is the point of origin for all microtubule arrays. This is because the MTOC contains a high concentration of γ-tubulin. Why is this important? With all of the cytoskeletal elements, though it is most pronounced with microtubules, the rate of nucleation, or starting a microtubule is significantly slower than the rate of elongating an existing structure. Since it is the same biochemical interaction, the assumption is that the difficulty lies in getting the initial ring of dimers into position. The γ-tubulin facilitates this process by forming a γ-tubulin ring complex that serves as a template for the nucleation of microtubules (fig. 6). This is true both in animal and fungal cells with a single defined MTOC, as well as in plant cells, which have multiple, dispersed sites of microtubule nucleation.

Transport on the Cytoskeleton

While it can be useful to think of these cytoskeletal structures as analogous to an animal skeleton, perhaps a better way to remember the relative placement of the microtubules and microfilaments is by their function in transporting intracellular cargo from one part of the cell to another. By that analogy, we might consider the microtubules to be a railroad track system, while the microfilaments are more like the streets. By the same analogy, we can suggest that the microtubule network and microfilament network are connected at certain points so that when cargo reaches its general destination by microtubule (rail), then it can be taken to its specific address by microfilament. Let's extend this analogy a bit further. If the microtubules and microfilaments are the tracks and streets, then what are the trains and trucks? Ah, an astute question, Grasshopper. On the microtubules, the "trains" are one of two families of molecular motors: the kinesins and the dyneins.

We can generalize somewhat and say that the kinesins drive towards the (+) end (toward periphery of cell) while the dyneins go toward the (-) end (toward the MTOC). On actin microfilaments, the molecular motors are proteins of the myosin family. At this point, the analogies end, as the functioning of these molecular motors is very different

from locomotion by train or truck. Finally, one might question the biological need for such a transport system. Again, if we analogize to human transport, then we could say that transport via simple diffusion is akin to people carrying packages randomly about the cell. That is to say, the deliveries will eventually be made, but you wouldn't want to count on this method for time-critical materials. Thus a directed, high-speed system is needed to keep cells (particularly larger, eukaryotic cells) alive.

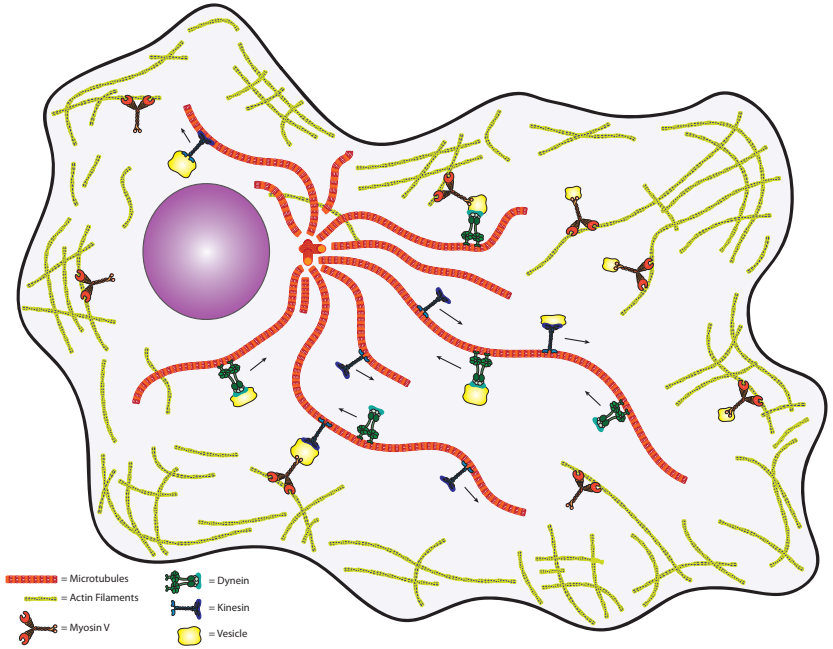


Figure 7. Transport on microtubules and microfilaments.

All of the kinesins and dyneins have a few key commonalities. There is a catalytic energy-releasing "head" connected to a hinge or neck region allowing the molecule to flex or "step", and there is a cargo-carrying tail beyond that (fig. 8). The head of a kinesin or dynein catalyzes the hydrolysis of ATP, releasing energy to change its conformation relative to the neck and tail of the molecule, allowing it to temporarily release its grip on the microtubule, swivel its "hips" around to plant itself a "step" away, and rebind to the microtubule (fig. 9). On the actin microfilaments, the myosins, of which there are also many types (some depicted in fig. 10) are the molecular motors. Their movement is different from dyneins and kinesins, as will be described in the next section, but also uses the energy of ATP hydrolysis to provide energy for the conformational changes needed for movement. We have introduced the motors, but considering the enormous diversity in the molecules that need to be transported around a cell, it would be impos-

Although this type of transport occurs in all eukaryotic cells, a particularly well-studied case is axonal transport (also called axoplasmic transport) in neurons. Here, the transport of materials from the cell body (soma) to the tips of the axons can sometimes traverse very long distances up to several meters in larger animals, and must do so in a timely manner. Axonal transport is generally classified as anterograde (from soma to axon terminal) or retrograde (from terminals back). The types of material transported in these two directions is very different: much of the anterograde transport is protein building blocks for extending the axon or synaptic vesicles containing neurotransmitters; retrograde transport is mostly endocytic vesicles and signaling molecules. Axonal transport is also categorized as fast and slow. Slow transport is primarily the movement of proteins directly bound to the motors, and they can move from from 100 μm per day (SCa, slow component a) up to 3mm/day (SCb). In comparison, fast transport is generally movement of vesicles, and can vary from 50 to 400 mm/day. The mechanism of slow transport had been debated for over a decade until 2000, when direct visualization of fluorescently labeled neurofilaments in transport showed that the actual movement of the proteins was very similar to the movement in fast axonal transport, but there were many pauses in the transport, a "stop and go" mechanism rather moving from source to destination continuously.

sible for the motors to directly bind to all of them. In fact, the motors bind to their cargo via adapter molecules that bind the motor on one side, and a cargo molecule or vesicle on the other. Further examination of the cargo and the routing of the cargo by address markers (SNAREs) was discussed in the vesicular transport chapter.

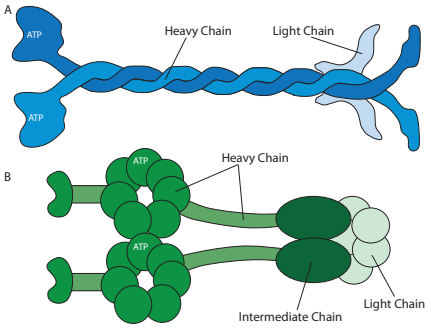


Figure 8. Kinesin (A) and Dynein (B) are motor proteins that move along microtubules. Generally, kinesins move to the (+) end while dyneins move to the (-) end. Their motor function requires ATP hydrolysis. ATP binding sites are marked in white.

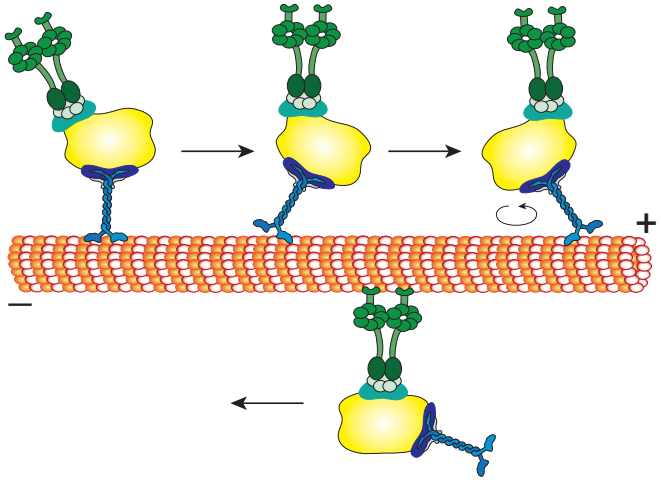


Figure 9. A cargo vesicle (yellow) can be simultaneously bound by dynein (green) and kinesin (blue) via adapter proteins. This top side also depicts the movement of the kinesin, in which binding of ATP causes one "foot" to release, and hydrolysis of ATP causes the molecule to swivel the other foot in front.

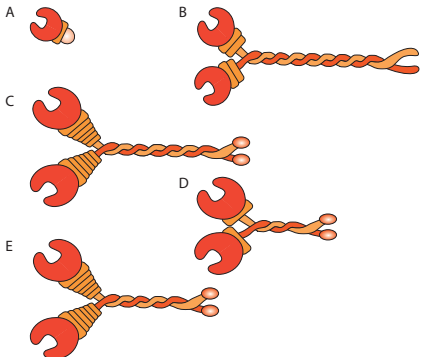


Figure 10. Selected Myosins. (A) Type I myosin, primarily for binding membranes to f-actin, including endocytic vesicles. (B) Type II myosin, binds f-actin on both ends to slide filaments against each other. (C) Type V myosin, used in vesicular transport. (D) Type VI myosin, used in endocytosis. (E) Type XI myosin, a fast myosin used in cytoplasmic streaming in plant cells.

Actin - Myosin Structures in Muscle

The motor proteins that transport materials along the acting microfilaments are similar in some ways, such as the globular head group that binds and hydrolyzes ATP, yet different in other ways, such as the motion catalyzed by the ATP hydrolysis. Much of the f-actin and myosin in striated and cardiac muscle cells is found in a peculiar arrangement designed to provide a robust contractile response over the entire length of the cell. The sarcomere is an arrangement of alternating fibers of f-actin (also known as "thin fibers" based on their appearance in electron micrographs) and myosin II (or "thick fibers"). Although we do not normally think of the motor protein as a fiber, in this case the tails of the myosin II molecules intertwine to form a continuous fiber of myosin molecules. As the contractile cycle proceeds, the myosin molecules grip the adjacent actin fibers, and move them. In fig. 11, you can see that a sarcomere is constructed so that the stationary myosin fibers are located centrally, with two parallel sets of actin fibers interspersed between the myosin fibers, to the left and the right of the center. Note that the actin fibers do not cross the center line, and that at the

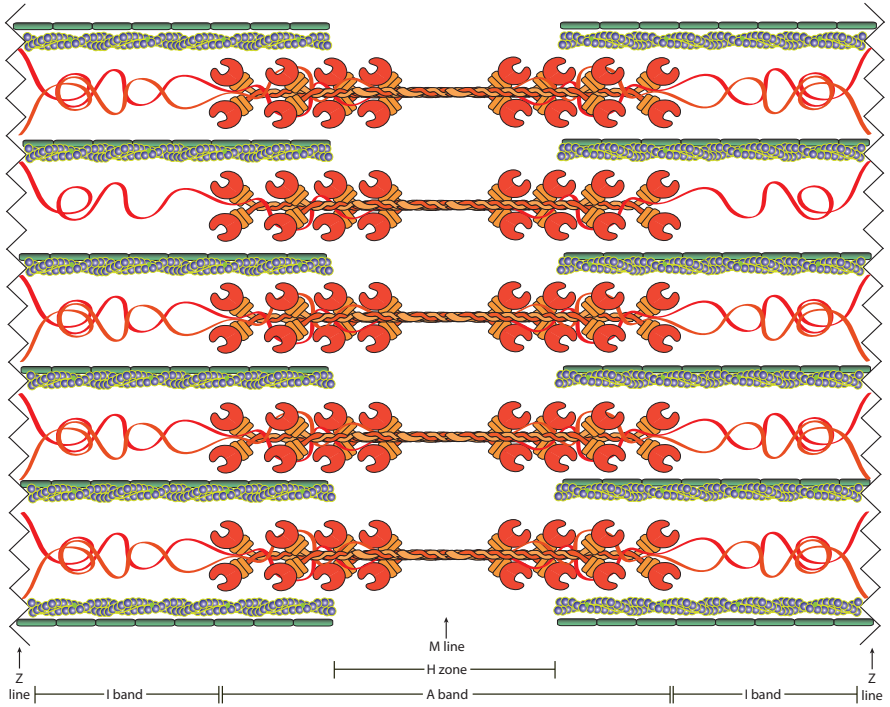


Figure 11. Sarcomere. Myosin II is depicted as in fig. 9, but here entwined with other myosins to form the thick filament. They are supported and anchored by titin (shown as long tangled orange ribbons). The myosin heads act on the actin filaments (blue), pulling them towards each other in a contractile movement.

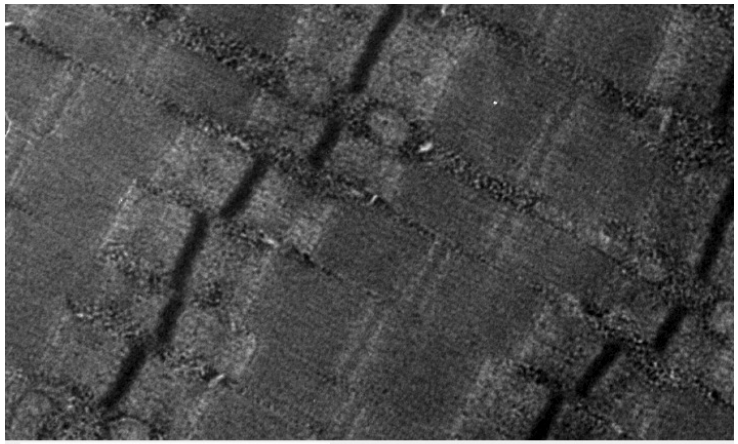


Figure 12. Human skeletal muscle is organized into sarcomeres. The dark Z lines are a clear reference point in comparing this to diagram in fig.11. This electron micrograph placed in the public domain by L. Howard.

center, the myosin molecules switch orientation. The physiological effect of this is that the actin filaments are all pulled inwards toward the center of the sarcomere. The sarcomere in turn, is merely one of many connected together to form a myofibril. The myofibrils extend the length of the muscle cell.

When the myosin head is in its resting state, it is tightly attached to the actin filament. In fact, *rigor mortis* occurs in dead animals because there is no more ATP being made, and thus the sarcomeres are locked into place. Rigor begins approximately 2-3 hours after death in humans, after reserves of ATP are depleted. When the body relaxes again in about 3 days, it is due to the decomposition and breakdown of the actin and myosin

OK, maybe you've watched CSI or Bones, etc enough to already know this, but pretty neat nonetheless, right?

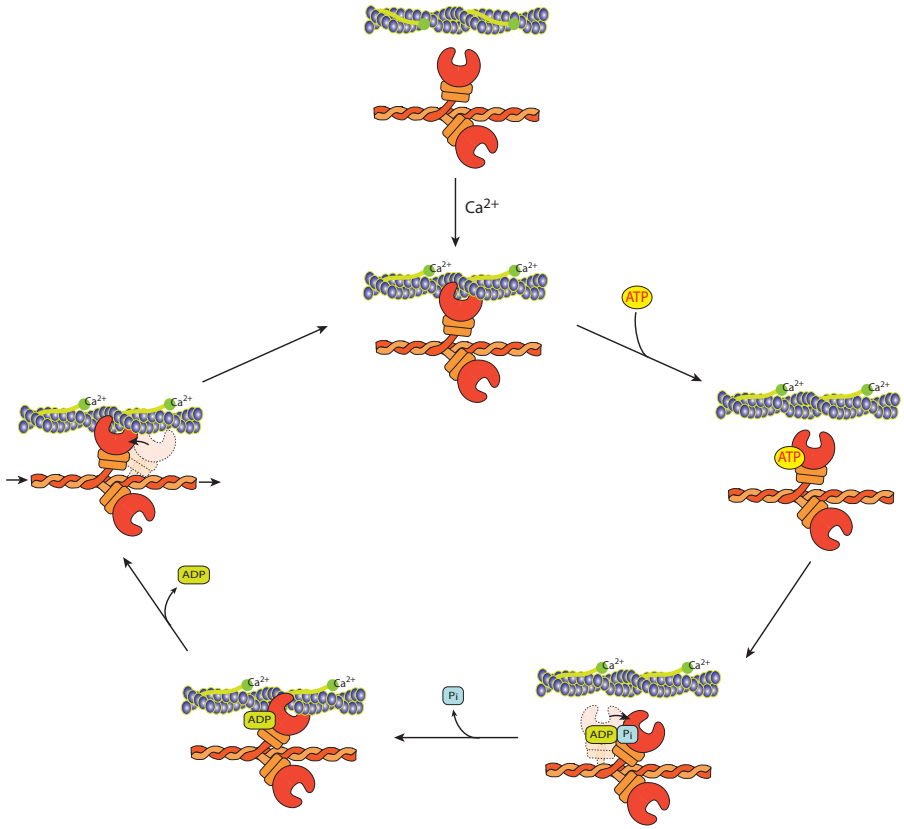


Figure 12. The myosin power stroke. Myosin can only attach to f-actin if there Ca^{++} available to bind troponin (green) and move tropomyosin (yellow) out of the binding groove. When ATP binds to the myosin head, it releases the f-actin. Hydrolysis of the ATP leads to cocking of the myosin head (moving it relative to the f-actin). As P_i leaves the myosin head, it reattaches to the f-actin, but slightly displaced from its original binding site. ADPis then released and the myosin undergoes a power stroke in which it springs back to its original position, moving the f-actin along with it.

proteins. However, while they are still living animals, ATP is generally available, and it can bind to the myosin head, causing it to lose affinity for the f-actin, and let go (fig. 12). At this point, no significant movement has occurred. Once the ATP is hydrolyzed though, the myosin head can reattach to the f-actin a little further down the filament than it had originally. The energy released is stored in the neck region. The ADP and P_i are still attached to the myosin head as well. The next step is for the P_i to drop off the myosin, leading to the power stroke. The neck of the myosin swivels around, leading to a translocation of the head by approximately 10 nm for myosin II. The distance of translocation varies depending on the type of myosin, but it is not yet clear whether the length of the neck is proportional to the displacement of the head. Finally, the ADP drops off the myosin head, increasing the affinity of the head for the f-actin.

The sarcomere structure described in the first paragraph was incomplete in order to place the major players clearly in their roles. There are other proteins in the sarcomere with important structural and regulatory functions. One of the key regulatory components is tropomyosin. This is a fibrous protein that lies in the groove of an actin microfilament and blocks access to the myosin binding site. Tropomyosin attaches to the microfilament in conjunction with a multi-subunit troponin complex. When Ca^{++} is available, it can bind to troponin-C, leading to a conformational change that shifts the position of tropomyosin to reveal the myosin binding site. This is the primary point of control for muscle contraction: recall that intracellular Ca^{++} levels are kept extremely low because its primary function is in intracellular signaling. One way that the Ca^{++} levels are kept that low is to pump it into a reservoir, such as the endoplasmic reticulum.

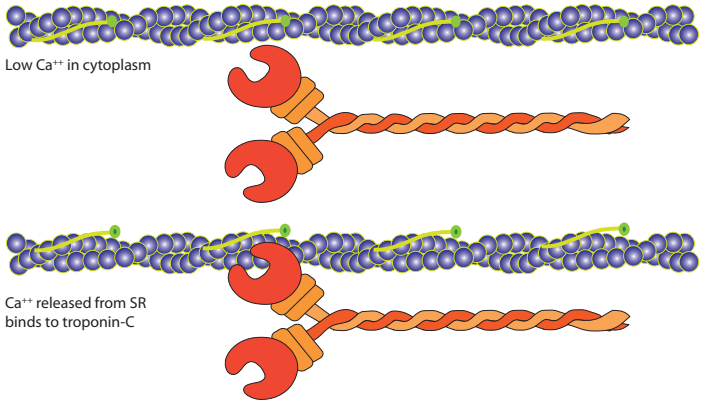


Fig. 13. In low Ca^{++} conditions, tropomyosin (yellow line) is held in the myosin-binding groove of f-actin (blue) by a tripartite troponin complex (light green). Once Ca^{++} levels increase, it can bind to troponin-C, causing a conformational shift that moves the tropomyosin out of the way so that myosin (orange) can bind the actin microfilaments.

In muscle cells, there is a specialization of the ER called the sarcoplasmic reticulum (SR) that is rich in Ca^{++} pumps and Ca^{++}. When a signal is sent from a controlling nerve cell to the muscle cell, it causes a depolarization of the muscle cell membrane. This consequently depolarizes a set of membranes called the transverse tubules (T-tubules) that lie directly on parts of the sarcoplasmic reticulum. There are proteins on the t-tubule surface that directly interact with a set of Ca^{++} channel proteins, holding the channel closed normally. When the t-tubule is depolarized, the proteins change shape, which changes the interaction with the Ca^{++} channels on the SR, and allows them to open. Ca^{++} rushes out of the SR where it is available to troponin-c. Troponin-C bound to Ca^{++} shifts the tropomyosin away from the actin filament, and the myosin head can bind to it. ATP can bind the myosin head to start the power stroke cycle, and voila, we have controlled muscle cell contraction.

In addition to the "moving parts", there are also more static, structural, proteins in the sarcomere (fig. 11). *Titin* is a gigantic protein (the largest known, at nearly 3 MDa), and can be thought of as something of a bungee cord tether to the myosin fiber. Its essential purpose is to prevent the forces generated by the myosin from pulling the fiber apart. Titin wraps around the myosin fiber and attaches at multiple points, with the most medial just near the edge of the H zone. At the Z-line, titin attaches to a telethonin complex, which attach to the Z-disk proteins (antiparallel α-actinin). Titin also interacts with obscurin in the I-band region, where it may link myofibrils to the SR, and in the M-band region it can interact with the Ca^{++}-binding protein calmodulin-1 and TRIM63, thought to acts as a link between titin and the microtubule cytoskeleton. There are multiple isoforms of titin from alternative splicing, with most of the variation coming in the I-band region.

Of course in an actual muscle (fig. 14), what happens is that nerves grow into the muscle and make synaptic connections with them. At these synaptic connections, the nerve cell releases neurotransmitters such as acetylcholine (ACh), which bind to receptors (AChR) on the muscle cell. This then opens ion channels in the muscle cell membrane, triggering a voltage change across that membrane, which also happens to affect the nearby membrane of the transverse tubules subsequently opening Ca^{++} channels in the SR. The contraction of sarcomeres can then proceed as already described above.

The SR is a specialization of part of the endoplasmic reticulum, and contains a high concentration of Ca^{++} ions because the SR membrane is embedded with Ca^{++} pumps (ATPases) to keep the cytoplasmic concentration low and sequester the Ca^{++} ions inside the SR. This is regulated by phosphorylation and $[Ca^{++}]$ via a regulatory protein such as phospholamban (in cardiac muscle). Phospholamban is an integral membrane protein of the SR that normally associates with and inhibits the Ca^{++} pump. However when it is phosphorylated, or as cytoplasmic Ca^{++} levels rise, the phospholamban releases from the Ca^{++} pump and allows it to function.

Disturbances to the proper formation of the titin-based support structure can be a cause of dilated cardiomyopathy, and from that, congestive heart failure. Some 20-30% of cases of dilated cardiomyopathy are familial, and mutations have been mapped to the N-terminal region of titin, where the protein interacts with telethonin. Defects in titin are also being investigated with respect to chronic obstructive pulmonary disease, and some types of muscular dystrophy.

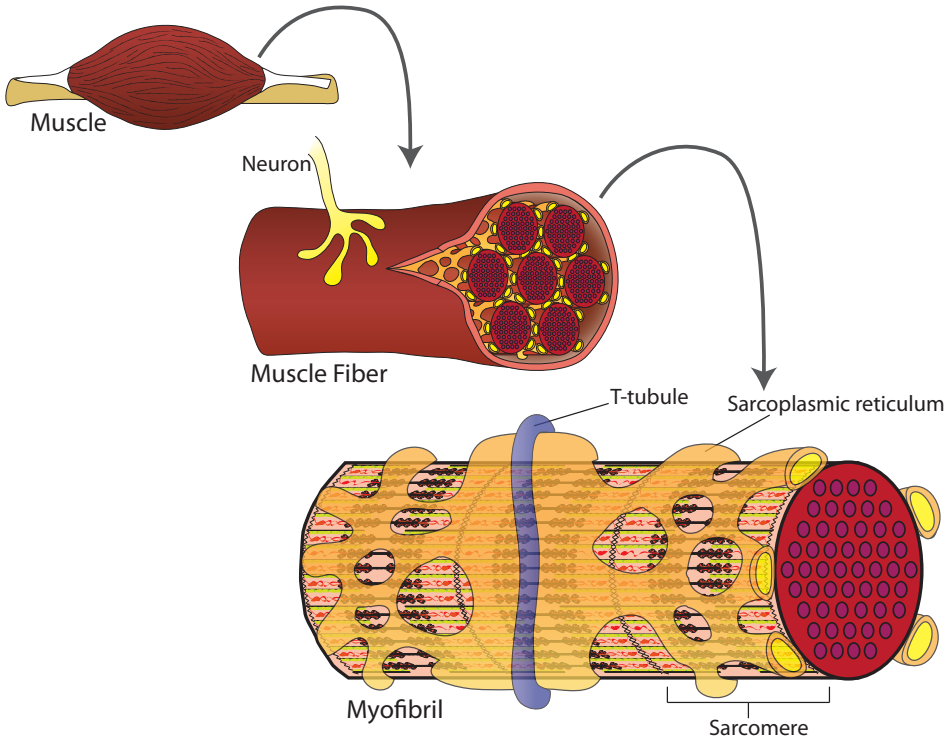


Figure 14. The sarcomere in context. The sarcomere of figures 11 and 12 is one tiny contractile unit within an array that forms a myofibril. The myofibril is one of many within a muscle cell, surrounded by the sarcoplasmic reticulum, a specialized extension of the ER that sequesters Ca^{++} until T-tubule excitation causes its release.

Cytoskeletal Dynamics

In the early development of animals, there is a huge amount of cellular rearrangement and migration as the roughly spherical blob of cells called the blastula starts to differentiate and form cells and tissues with specialized functions. These cells need to move from their point of birth to their eventual positions in the fully developed animal. Some cells, like neurons, have an additional type of cell motility - they extend long processes (axons) out from the cell body to their target of innervation. In both neurite extension and whole cell motility, the cell needs to move first its attachment points and then the bulk of the cell from one point to another. This is done gradually, and uses the cytoskeleton to make the process more efficient. The major elements in cell motility are changing the point of forward adhesion, clearing of internal space by

myosin-powered rearrangement of actin microfilaments and the subsequent filling of that space with microtubules.

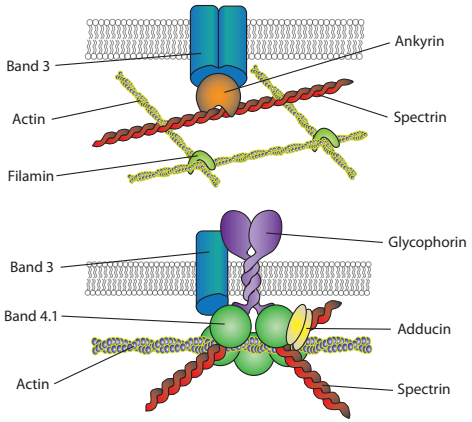


Figure 15. Membrane to microfilament linkage complexes in erythrocytes involve spectrin.

For force to be transmitted, the membrane must be attached to the cytoskeleton. In fact, signaling (chap. 14) from receptors in the membrane can sometimes directly induce rearrangements or movements of the cytoskeleton via adapter proteins that connect actin (or other cytoskeletal elements) to transmembrane proteins such as integrin receptors. One of the earliest experimental systems for studies of cytoskeleton-membrane interaction was the erythrocyte (red blood cell). The illustrations at right (fig. 15) show some of the interactions of an extensive actin microfilament network with transmembrane proteins. Ankyrin and spectrin are important linkage proteins between the transmembrane proteins and the microfilaments. This idea of building a protein complex around the cytoplasmic side of a transmembrane protein is ubiquitous, and scaffolding (linking) proteins are used not only in connecting the extracellular substrate (via transmembrane protein) to the cytoskeleton, but also to physically connect signaling molecules and thus increase the speed and efficiency of signal transduction.

Accessory proteins to actin filaments and microtubules were briefly mentioned earlier. Among other functions, they can control polymerization and depolymerization, form bundles, arrange networks, and bridge between the different cytoskeletal networks. For actin, the primary polymerization control proteins are profilin, which promotes polymerization and thymosin β_4, which sequesters g-actin. The minus end capping proteins Arp 2/3 complex and tropomodulin, and the plus end capping proteins CapZ, severin, and gelsolin can stabilize the ends of f-actin. Finally, cofilin can increase depolymerization from the (-) end.

Profilin has two activities that promote polymerization. First, it is a nucleotide exchange factor that removes ATP bound to g-actin, and replaces it with ADP. This sounds counterintuitive, but keep reading through to the next paragraph. Second, when bound to a g-actin, it increases the rate of addition to actin microfilaments. It does so by binding to the end opposite the ATP-binding site, leaving that site and that side open to binding both ATP and the (+) end of a microfilament. Profilin can be found

both in the cytoplasm at large, and associated with phospholipids (PIP_2) and membrane proteins, to control such processes as leading edge remodeling of f-actin cytoskeletal structures.

Thymosin β_4 regulates microfilament assembly by controlling the available pool of g-actin. We already stated that greater concentrations of g-actin can increase polymerization rates. However, because of the highly dynamic nature of the actin cytoskeleton, the time constraints of degrading and producing new actin would prevent the fast-response control necessary. Therefore, the optimal mechanism is to maintain a large pool of g-actin monomers, but regulate its availability by tying it up with a sequestering protein - thymosin β_4. Thymosin β_4 has a 50x higher affinity for g-actin-ATP than for g-actin-ADP, so here is where profilin comes back into the picture. Profilin exchanges the ATP of a $T\beta_4$-g-actin-ATP complex for an ADP. The result is that the $T\beta_4$ releases the g-actin-ADP, allowing it to enter the general pool for building up filaments.

Increased depolymerization and slowing or cessation of polymerization can gradually break down f-actin structures, but what if there is a need for rapid breakdown? Two of the capping proteins previously mentioned, gelsolin and severin, have an alternate mode of action that can sever actin microfilaments at any point by binding alongside an actin filament and altering the conformation of the subunit to which it is bound. The conformational change forces the actin-actin interaction to break, and the gelsolin or severin then remains in place as a (+) end capping protein.

Gelsolin is inhibited by the phospholipid PIP_2. Phospholipase C, which breaks down PIP_2 can also increase cytosolic Ca^{++}, which is an activator of gelsolin. Thus it is possible to rapidly upregulate gelsolin activity by PLC signaling.

On the microtubule side of things, due to dynamic instability, one might think that a severing enzyme is not needed, but in fact, spastin and katanin are microtubule-severing proteins found in a variety of cell types, particularly neurons. There is also a $T\beta_4$-like protein for tubulin: Op18, or stathmin, which binds to tubulin dimers (not monomers), acting to sequester them and lower the working concentration. It is regulated by phosphorylation (which turns off its tubulin binding). Microtubule-associated proteins MAP1, MAP2, and tau (τ) each work to promote assembly of microtubules, as well as other functions. MAP1 is the most generally distributed of the three, with tau being found mostly in neurons, and MAP2 even more restricted to neuronal dendrites. These and some other MAPs also act to stabilize microtubules against catastrophe by binding alongside the microtubule and reinforcing the tubulin-tubulin interactions.

Mutations in spastin are linked to 40% of those spastic paraplegias distinguished by degeneration of very long axons. The severing ability of spastin appears to be required for remodeling of the cytoskeleton in response to neuronal damage.

Tau has a complicated biomedical history. Its normal function is clear - assembling, stabilizing, and linking microtubules. However, it is also found in hyperphosphorylated neurofibrillary tangles that are associated with Alzheimer's disease. A cause for Alzheimer's is not yet known, so it is still unclear whether the tau protein tangles are play a major role in any of the symptoms.

Finally, with respect to microfilament and microtubule accessory proteins, there are the linkers. Some of the aforementioned MAPs can crosslink microtubules either into parallel or mesh arrays, as can some kinesins and dyneins, although they are conventionally considered to be motor proteins. On the microfilament side, there are many known proteins that crosslink f-actin, many of which are in the calponin homology

domain superfamily, including fimbrin, α-actinin, b-spectrin, dystrophin, and filamin. Although they all can bind to actin, the shape of the protein dictates different types of interaction: for example, fimbrin primarily bundles f-actin in parallel to form bundles, while filamin brings actin filaments together perpendicularly to form mesh networks.

Cell Motility

There are a number of ways in which a cell can move from one point in space to another. In a liquid medium, that method may be some sort of swimming, utilizing ciliary or flagellar movement to propel the cell. On solid surfaces, those mechanisms clearly will not work efficiently, and the cell undergoes a crawling process. In this section, we begin with a discussion of ciliary/flagellar movement, and then consider the more complicated requirements of cellular crawling.

Cilia and flagella, which differ primarily in length rather than construction, are microtubule-based organelles that move with a back-and-forth motion. This translates to "rowing" by the relatively short cilia, but in the longer flagella, the flexibility of the structure causes the back-and-forth motion to be propagated as a wave, so the flagellar movement is more undulating or whiplike (consider what happens as you waggle a garden hose quickly from side to side compared to a short piece of the same hose). The core of either structure is called the axoneme, which is composed of 9 microtubule doublets connected to each other by *ciliary dynein* motor proteins, and surrounding a central core of two separate microtubules. This is known as the "9+2" formation, although the nine doublets are not the same as the two central microtubules. The A tubule is a full 13-protofilaments, but the B tubule fused to it contains only 10 protofilaments. Each of the central microtubules is a full 13 protofilaments. The 9+2 axoneme extends the length of the cilium or flagellum from the tip until it reaches the base, and connects to the cell body through a *basal body*, which is composed of 9 microtubule triplets arrange in a short barrel, much like the centrioles from which they are derived.

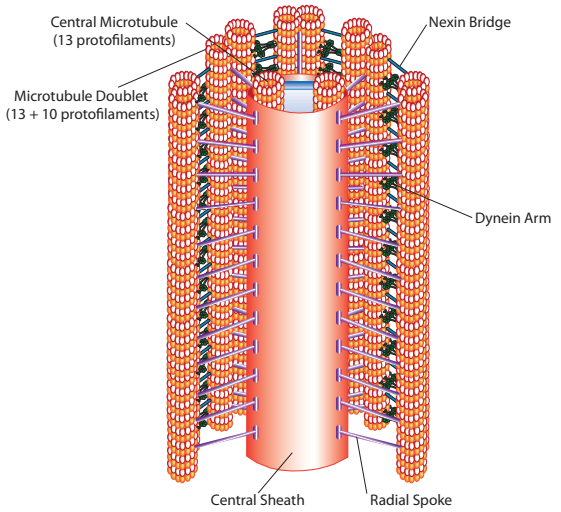


Figure 16. Partial (cutaway) diagram of an axoneme, the central bundle of cilia and flagella.

FG Syndrome is a genetically linked disease characterized by mental retardation, enlarged head, congenital hypotonia, imperforate anus, and partial agenesis of the corpus callosum. It has been linked to mutations in several X chromosome genes, including filamin A (FLNA, FLN1, located Xq28).

Mutations in dystrophin, which is a major muscle protein of the CD-domain superfamily, can result in Duchenne Muscular Dystrophy or the related but less severe Becker Muscular Dystrophy. The most distinctive feature is a progressive proximal muscular degeneration and pseudohypertrophy of the calf muscles. Onset of DMD is usually recognized before age 3 and is lethal by age 20. However, symptoms of BMD may not present until the 20s, with good probability of long-term survival. Although it is primarily a muscle-wasting disease, dystrophin is present in other cell types, including neurons, which may explain a link to mild mental retardation in some DMD patients. Like FLNA, the dystrophin gene is also located on the X chromosome (Xp21.2).

The ciliary dyneins provide the motor capability, but there are two other linkage proteins in the axoneme as well. There are *nexins* that join the A-tubule of one doublet to the B-tubule of its adjacent doublet, thus connecting the outer ring. And, there are *radial spokes* that extend from the A tubule of each doublet to the central pair of microtubules at the core of the axoneme. Neither of these has any motor activity. However, they are crucial to the movement of cilia and flagella because they help to transform a sliding motion into a bending motion. When ciliary dynein (very similar to cytoplasmic dyneins but has three heads instead of two) is engaged, it binds an A microtubule on one side, a B microtubule from the adjacent doublet, and moves one relative to the other. A line of these dyneins moving in concert would thus slide one doublet relative to the other, if (and it's a big "if") the two doublets had complete freedom of movement. However, since the doublets are interconnected by the nexin proteins, what happens as one doublet attempts to slide is that it bends the connected structure instead (fig. 17). This bend accounts for the rowing motion of the cilia, which are relatively short, as well as the whipping motion of the long flagella, which propagate the bending motion down the axoneme.

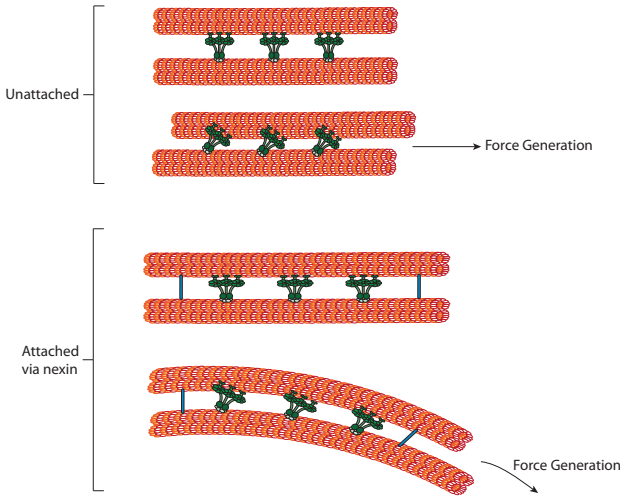


Figure 17. The nexin bridges connecting adjacent microtubule doublets transform the sliding motion generated by the ciliary dynein into a bending motion.

Although we think of ciliary and flagellar movement as methods for the propulsion of a cell, such as the flagellar swimming of sperm towards an egg, there are also a number of important places in which the cell is stationary, and the cilia are used to move fluid past the cell. In fact, there are cells with cilia in most major organs of the body. Several ciliary dyskinesias have been reported, of which the most prominent, primary ciliary dyskinesia (PCD), which includes Kartagener syndrome (KS), is due to mutation

This section refers only to eukaryotes. Some prokaryotes also have motile appendages called flagella, but they are completely different in both structure and mechanism. The flagella themselves are long helical polymers of the protein flagellin, and the base of the flagellin fibers is connected to a rotational motor protein, not a translational motor. This motor (fig. 18) utilizes ion (H^+ or Na^+ depending on species) down an electrochemical gradient to provide the energy to rotate as many as 100000 revolutions per minute. It is thought that the rotation is driven by conformational changes in the stator ring, nestled in the cell membrane.

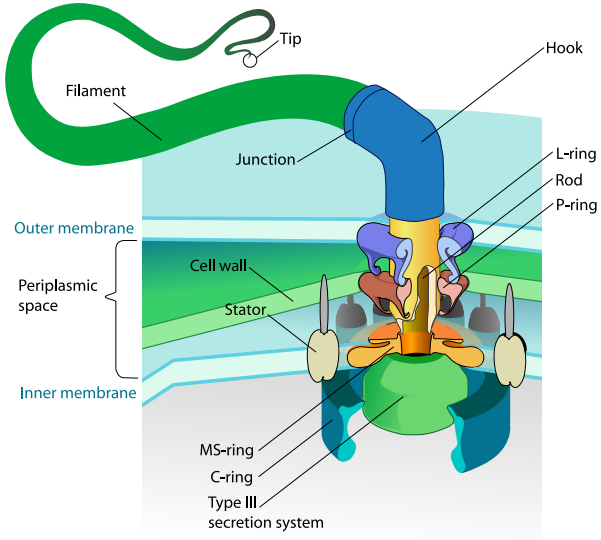


Figure 18. The bacteria flagellum is completely different from eukaryotic flagella. It is moved by a rotary motor driven by proton or Na^+ ion flow down the electrochemical gradient. Illustration released to public domain by M.R. Villareal.

of the DNAI1 gene, which encodes a subunit (intermediate chain 1) of axonemal (ciliary) dynein. PCD is characterized by respiratory distress due to recurrent infection, and the diagnosis of KS is made if there is also *situs inversus*, a condition in which the normal left-right asymmetry of the body (e.g. stomach on left, liver on right) is reversed. The first symptom is due to inactivity of the numerous cilia of epithelial cells in the lungs. Their normal function is to keep mucus in the respiratory track constantly in motion. Normally the mucus helps to keep the lungs moist to facilitate function, but if the mucus becomes stationary, it becomes a breeding ground for bacteria, as well as becoming an irritant and obstacle to proper gas exchange.

Situs inversus is an interesting malformation because it arises in embryonic development, and affects only 50% of PCD patients because the impaired ciliary function causes randomization of left-right asymmetry, not reversal. In very simple terms, during early embryonic development, left-right asymmetry is due in part to the movement of molecular signals in a leftward flow through the embryonic node. This flow is caused by the coordinated beating of cilia, so when they do not work, the flow is disrupted and randomization occurs.

Other symptoms of PCD patients also point out the work of cilia and flagella in the body. Male infertility is common due to immotile sperm. Female infertility, though less common, can also occur, due to dysfunction of the cilia of the oviduct and fallopian tube that normally move the egg along from ovary to uterus. Interestingly there is also a low association of hydrocephalus internus (overfilling of the ventricles of the brain with cerebrospinal fluid, causing their enlargement which compresses the brain tissue around them) with PCD. This is likely due to dysfunction of cilia in the ependymal cells lining the ventricles, and which help circulate the CSF, but are apparently not completely necessary. Since CSF bulk flow is thought to be driven primarily by the systole/diastole change in blood pressure in the brain, some hypothesize that the cilia may be involved primarily in flow through some of the tighter channels in the brain.

Cell crawling (fig. 19) requires the coordinated rearrangement of the leading edge microfilament network, extending (by both polymerization and sliding filaments) and then forming adhesions at the new forward-most point. This can take the form of filopodia or lamellipodia, and often both simultaneously. *Filopodia* are long and very thin projections with core bundles of parallel microfilaments and high concentrations of cell surface receptors. Their purpose is primarily to sense the environment. *Lamellipodia* often extend between two filopodia and is more of a broad ruffle than a finger. Internally the actin forms more into meshes than bundles, and the broader edge allows for more adhesions to be made to the substrate. The microfilament network then rearranges again, this time opening a space in the cytoplasm that acts as a channel for the movement of the microtubules towards the front of the cell. This puts the transport network in place to help move intracellular bulk material forward. As this occurs, the old adhesions on the tail end of the cell are released. This release can happen through two primary mechanisms: endocytosis of the receptor or deactivation of the receptor by signaling/conformational change. Of course, this oversimplification belies the complexities in coordinating and controlling all of these actions to accomplish directed movement of a cell.

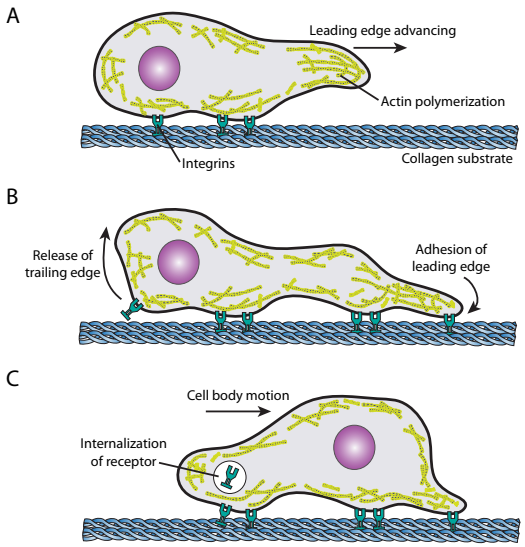


Figure 19. Cells crawl by (a) extending the leading edge primarily through remodeling of the actin cytoskeleton, (b) forming new adhesive contacts at that leading edge while releasing adhesions to the rear, and (c) bulk internal movement forward to "catch up" with the leading edge.

Once a cell receives a signal to move, the initial cytoskeletal response is to polymerize actin, building more microfilaments to incorporate into the leading edge. Depending on the signal (attractive or repulsive), the polymerization may occur on the same or opposite side of the cell from the point of signal-receptor activation. Significantly, the polymerization of new f-actin alone can generate sufficient force to move the membrane forward, even without involvement of myosin motors! Models of force generation are being debated, but generally start with the incorporation of new g-actin into a filament at its tip; that is, at the filament-membrane interface. Even if that might technically be enough, in a live cell, myosins are involved, and help to push and arrange filaments directionally in order to set up the new leading edge. In addition, some

One model of microfilament force generation, the Elastic Brownian Ratchet Model (Mogilner and Oster, 1996), proposes that due to Brownian motion of the cell membrane resulting from continuous minute thermal fluctuation, the actin filaments that push out towards the edges of the membrane are flexed to varying degrees. If the flex is large enough, a new actin monomer can fit in between the membrane and the tip of the filament, and when the now longer filament flexes back, it can exert a greater push on the membrane. Obviously a single filament does not geneate much force, but the coordinated extension of many filaments can push the membrane forward.

filaments and networks must be quickly severed, and new connections made, both between filaments and between filaments and other proteins such as adhesion molecules or microtubules.

How is the polymerization and actin rearrangement controlled? The receptors that signal cell locomotion may initiate somewhat different pathways, but many share some commonalities in activating one or more members of the Ras-family of small GTPases. These signaling molecules, such as Rac, Rho, and cdc42 can be activated by receptor tyrosine kinases (see RTK-Ras activation pathways, Chap. 14). Each of these has a slightly different role in cell motility: cdc42 activation leads to filopodia formation, Rac activates a pathway that includes Arp2/3 and cofilin to lamellipodia formation, and Rho activates myosin II to control focal adhesion and stress fiber formation. A different type of receptor cascade, the G-protein signaling cascade (also Chapter 14), can lead to activation of PLC and subsequent cleavage of PIP_2 and increase in cytosolic Ca^{++}. These changes, as noted earlier, can also activate myosin II, as well as the remodeling enzymes gelsolin, cofilin, and profilin. This breaks down existing actin structures to make the cell more fluid, while also contributing more g-actin to form the new leading edge cytoskeleton.

In vitro experiments show that as the membrane pushes forward, new adhesive contacts are made through adhesion molecules or receptors that bind the substrate (often cell culture slides or dishes are coated with collagen, laminin, or other extracellular matrix proteins). The contacts then recruit cytoskeletal elements for greater stability to form a focal adhesion (fig. 20). However, the formation of focal adhesions appears to be an artifact of cell culture, and it is unclear if the types of adhesions that form in vivo recruit the same types of cytoskeletal components.

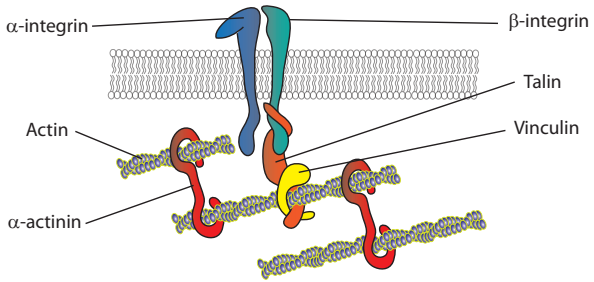


Figure 20. Focal adhesions form when integrins bind to an artificial ECM surface in cell culture dishes.

The third step to cell locomotion is the bulk movement of the cellular contents forward. The mechanisms for this phase are unclear, but there is some evidence that using linkages between the actin cytoskeleton at the leading edge and forward parts of the microtubule cytoskeleton, the microtubules are rearranged to form an efficient

transport path for bulk movement. Another aspect to this may be a "corralling" effect by the actin networks, which directionally open up space towards the leading edge. The microtubules then enter that space more easily than working through a tight actin mesh, forcing flow in the proper direction.

Finally, the cell must undo its old adhesions on the trailing edge. This can happen in a number of different ways. In vitro, crawling cells have been observed to rip themselves off of the substrate, leaving behind tiny bits of membrane and associated adhesion proteins in the process. The force generated is presumed to come from actin-myosin stress fibers leading from the more forward focal adhesions. However, there are less destructive mechanisms available to the cells. In some cases, the adhesivity of the cellular receptor for the extracellular substrate can be regulated internally, perhaps by phosphorylation or dephosphorylation of a receptor. Another possibility is endocytosis of the receptor, taking it off the cell surface. It could simply recycle up to the leading edge where it is needed (i.e. transcytosis), or if it is no longer needed or damaged, it may be broken down in a lysosome.

Much of the work on microtubule-actin interactions in cell motility has been done through research on the neuronal growth cone, which is sometimes referred to as a cell on a leash, because it acts almost independently like a crawling cell, searching for the proper pathway to lead its axon from the cell body to its proper synaptic connection (A.W. Schaefer et al, Dev. Cell 15: 146-62, 2008).

ECM and Adhesion :

Cell-Matrix and Cell-Cell Adhesion

Using this book: This book is designed to be used in both introductory and advanced cell biology courses. The primary text is generally on the left side of the vertical divider, and printed in black. Details that are usually left to an advanced course are printed in blue and found on the right side of the divider. Finally, additional biomedically relevant information can be found in red print on either side of the divider.

Interactions between a cell and its environment or with other cells are governed by cell-surface proteins. This chapter examines a subset of those interactions: direct cell contact with either other cells or extracellular matrix (ECM). Extracellular matrix is a general term for the extremely large proteins and polysaccharides that are secreted by some cells in a multicellular organism, and which acts as connective material to hold cells in a defined space. Cell density can vary greatly between different tissues of an animal, from tightly-packed muscle cells with many direct cell-to-cell contacts to liver tissue, in which some of the cells are only loosely organized, suspended in a web of extracellular matrix.

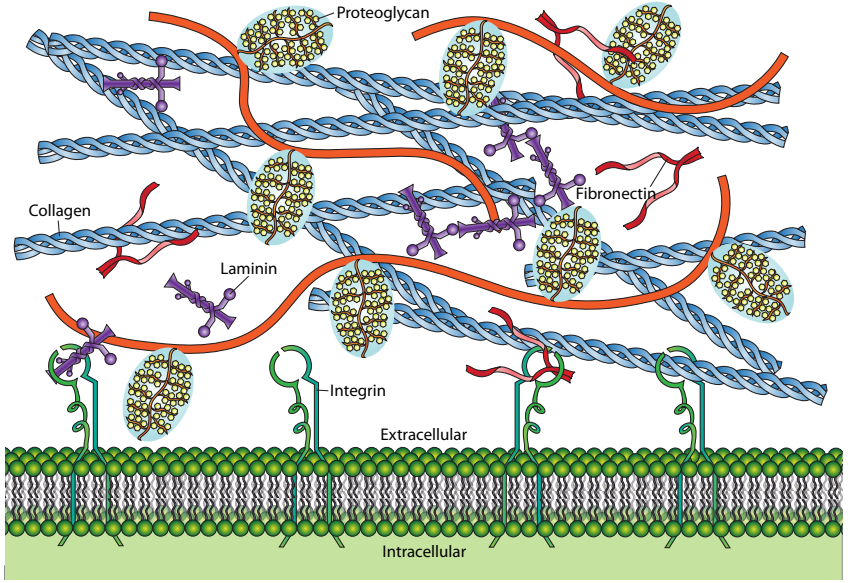


Figure 1. Extracellular matrix (ECM). Typical components include collagen, proteoglycans (with hydration shell depicted around sugars), fibronectin, and laminin. The cellular receptors for a number of these ECM components are integrins, although the exact integrin αβ pair may differ.

ECM is a generic term encompassing mixtures of polysaccharides and proteins, including collagens, fibronectins, laminins, and proteoglycans, all secreted by the cell. The proportions of these components can vary greatly depending on tissue type. Two, quite different, examples of ECM are the basement membrane underlying the epidermis of the skin, a thin, almost two-dimensional layer that helps to organize the skin cells into a nearly-impenetrable barrier to most simple biological insults, and the massive three-dimensional matrix surrounding each chondrocyte in cartilaginous tissue. The ability of the cartilage in your knee to withstand the repeated shock of your footsteps is due to the ECM proteins in which the cells are embedded, not to the cells that are actually rather few in number and sparsely distributed. Although both types of ECM share some components in common, they are clearly distinguishable not just in function or appearance, but in the proportions and identitiy of the constituent molecules.

Collagen

The largest and most prominent of the extracellular matrix proteins, constituting a quarter of the dry mass of the human body, are the members of the collagen family. Collagens are polymers that can be categorized into fibrillar (e.g. collagens I, II, III) and nonfibrillar (e.g. collagen IV) types. The fibrillar collagens are made up of triple helical monomers of either identical (homotrimer) or different (heterotrimer) subunits. These monomers are then associated in an offset parallel interaction with other collagen monomers, leading to the formation of long fibers. Electron microscopic examination of these long fibers shows a banding pattern, which is indicative of the slight gap between monomers along the same parallel.

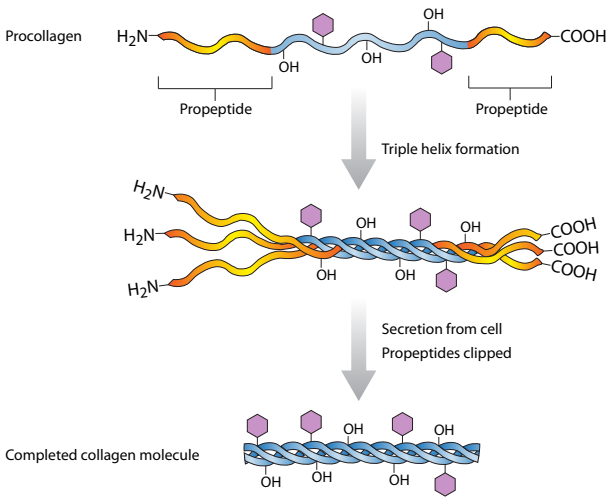


Figure 2. Collagen is a triple-helical protein consisting of three fibrillar subunits. Some of the amino acids are hydroxylated (see fig. 3), and the protein is also glycosylated (represented by purple hexagons).

The "basal lamina" and "basement membrane" are frequently confused by students and professionals alike. The basement membrane was discovered first as a very thin layer of connective proteins just beneath an epithelial cell layer. The basal lamina was not discovered until later because it is not visible by light microscopy (normally only ~50 nm thick). Technically, the basal lamina, which consists of multiple layers itself, is a layer of ECM proteins secreted by the epithelial layer. The basal lamina and a thick reticular lamina (ECM secreted by other cell types) together form what is considered the basement membrane.

The basal lamina around glomerular blood vessels in the kidneys is twice as thick (up to 100 nm) as usual, accomplishing part of the kidneys' physiological role in blood filtration.

Like all secreted proteins, collagen I is processed in the ER (fig. 2), but not completely assembled there: the three pro-α-chains are assembled into a procollagen triple helix, which is secreted. Extracellularly, they must then be cleaved at both termini to form the active collagen protein, which is completely fibrillar. Other collagen types do not have the same cleavage, and may have globular domains at the ends of the fibrils. Collagens are also interesting for their unusual amino acid makeup. They contain a high proportion of hydroxylated amino acids, mostly prolines and lysines (fig. 3). This hydroxylation is necessary for the extensive hydrogen bonding that occurs between subunits and between monomers. The fibrils are associated with high tensile strength. An example of this would be the long collagen fibers that run parallel to the long axis of tendons and ligaments. These high-stress-bearing structures (connecting bone to muscle, and bone to bone, respectively) require the resilience that collagen fibers can provide.

Conversely, conditions that adversely affect collagen formation can lead to serious disease conditions. In fact, a form of epidermolysis bullosa (the heritable skin blistering disease introduced in the previous chapter) is caused by mutation in collagen VII which is primarily produced by epidermal keratinocytes and secreted into the dermal-epidermal basement membrane layer. A variety of chondrodysplasias as well as bone malformations such as osteogenesis imperfecta (which can be perinatally lethal) have been linked to mutations in various collagen genes. Finally, several symptoms of scurvy are due to malformation of collagen in the ECM: weak blood vessel walls, bleeding gums and loose teeth, and fragile bones. Scurvy is a disease of ascorbic acid (vitamin C) deficiency, and the effect on ECM is due to the need for ascorbic acid as a cofactor for enzymes that hydroxylate the prolines and lysines of collagen.

Collagen is a major component of the basement membrane and basal lamina. The basal lamina is strong and flexible, able to serve as structural support for the epithelial sheets attached to it, as well as providing a semi-permeable matrix/filter that allows the passage of water and smaller molecules, but excludes larger macromolecules. The two major protein components to the basal lamina are collagen IV and laminin. Collagen IV has both long fibrillar, alpha-helical domains as well as globular domains that can interact in different orientations to form the meshwork that sets up the basement membrane. The laminin network is connected to the collagen network through entactin (nidogen) linker proteins.

An interesting application of collagen fibrils is in the cornea, the protective clear covering of the eye. The cornea is the primary protection against eye injury, and must be tough. The central layer (stroma, or substantia propria) is composed of approximately 200 layers of tightly packed, regularly spaced parallel collagen fibrils, with adjacent lay-

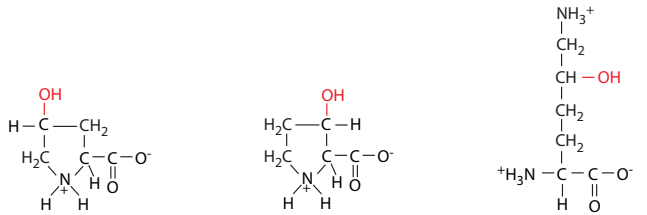


Figure 3. Collagens have a high proportion of hydroxylated prolines and lysines.

ers arranged so that the collagen fibrils lie perpendicularly from one layer to the next. This kind of laminar structure is used in a variety of man-made construction materials (including the ubiquitous building material, plywood) and provides great strength in a relatively small mass. Somewhat amazingly, and quite unlike plywood, the cornea is transparent. That property is thought to come from the regularity of the collagen lattice, which allows for cancellation of scattered light from one fibril by destructive interference from the scattered light of another fibril. Somewhat counterintuitively, it actually gets cloudy (due to refraction) when it absorbs fluid from the aqueous humor, and has active mechanisms to pump any such fluid back out of the cornea. This is why the cornea thickens and becomes translucent after death – the pump mechanism no longer has energy to run, and the aqueous humor diffuses into the cornea.

Proteoglycans

The protein component of proteoglycans are not as large as fibrillar collagens in general, but they often fill a massive volume because of heavy glycosylation. The sugars, many of which are sulfated or carboxylated, are hygroscopic to begin with, but being negatively charged, attract positive ions, which in turn brings in more water. Sugars attached to the core proteins are usually repeating disaccharide units such as chondroitin (D-Glucuronic acid and GalNAc), chondroitin sulfate, heparin (D-Glucuronic acid and GlcNAc by α/β1-4 bond), heparan sulfate, keratan sulfate (Galactose and GlcNAc), or hyaluronan (also called hyaluronic acid, composed of D-Glucuronic acid linked by β1-3 bond to GlcNAc). As with all glycoproteins, assembly of the GAGs occurs in the Golgi,

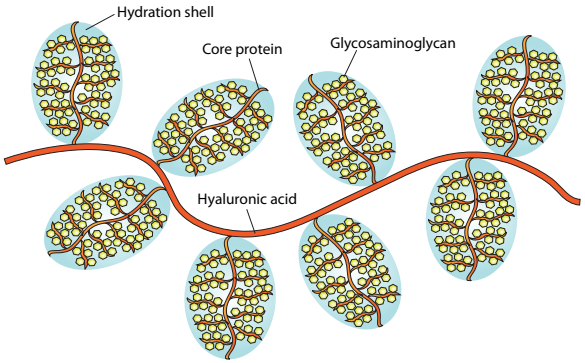


Figure 4. Proteoglycans are composed of multiple glycosaminoglycans attached to a core protein. These core proteins are sometimes attached to a hyaluronic acid molecule. Negatively charged sugars, like chondroitin sulfate or heparan sulfate, are depicted in yellow. They attract positive ions and water, forming a hydration shell around the proteoglycan. This figure depicts aggrecan, a cartilaginous aggregate of proteoglycans assembled on a hyaluronic acid core.

but beyond that, mechanisms for control of the extent and length of the disaccharide polymer addition is unknown. Unlike collagens and most other ECM components, proteoglycans can either be secreted or membrane bound. In fact, of the membrane

The reasoning behind the use of glucosamine and chondroitin sulfate supplements by people with joint problems is that they are two of the sugars found in proteoglycans of cartilaginous tissue such as the meniscus of the knee, and in other joints. Chondroitin sulfate in particular is the major sugar in articular cartilage proteoglycans. Both are thought to stimulate GAG synthesis, and limite documentation of protease inhibitory and collagen synthesis effects have been noted. Data from rabbit models (but potential conflict of interest, Lippiello et al, 2000) suggests a therapeutic benefit from such supplements. However, human studies have so far shown no significant improvement in patients already suffering from moderate to severe arthritis and other joint-related ailments (Clegg et al, 2006). A secondary survey analysis suggested that there was some promise with regard to effects on mild to moderate cases, but the data was not significant.

Heparin, a hypersulfated form of heparan sulfate, is also used medically as an anticlotting drug. It does so not by preventing clots directly, but by activating antithrombin III, which inhibits clotting.

bound proteoglycans, some are actually transmembrane proteins (these are designated syndecans), while other are bound to the cell surface via glycosylphosphatidylinositol (GPI) anchor (glypicans). In addition to these three basic varieties of core proteins, proteoglycans exhibit extraordinary diversity in glycosylation, ranging from the addition of only a few sugars, to well over a hundred. Interestingly, the core protein for chondroitin sulfate proteoglycans in basal lamina of muscle can be a collagen (Type XV)!

One of the paradoxes of proteoglycans is that they can function either as a substrate for cells to attach to, or due to the hydration shell, they can be very effective barriers to other cells as well. This is useful during development when there is a great deal of cell migration, and there needs to be ways to segregate cells both by attracting them and repelling them. Unfortunately, this can have deleterious consequences in some situations. For example, when the brain or spinal cord is injured, a glial scar is formed, and that scar contains a chondroitin sulfate proteoglycan. Unfortunately, this proteoglycan is an inhibitor of neural growth, which contributes to the prevention of neural regeneration, and for the unlucky patient, likely paralysis or worse depending on location and severity of the lesion.

On the other hand, there is also evidence (Rolls et al, *PLoS Med.* 5: e171, 2008) that the CSPG may be needed to activate microglia and macrophages to promote healing.

Fibronectins

Fibronectin and laminin are significantly smaller than either collagens or proteoglycans, and play different roles in the extracellular matrix. Fibronectin is formed by the joining of two similar polypeptide subunits via a pair of disulfide bonds near the C-terminal of each (fig. 5). Each subunit is arranged as a linear sequence of 30 functional domains (varies slightly by species). Within each subunit, each domain acts as a semi-independent unit with respect to secondary and even tertiary structure. Structurally, there are three major types of domains (Fig. 6) that can be distinguished not only by sequence, but by the binding sites they form. The figure above shows binding sites for other fibronectins, fibrin, collagen, heparin, and syndecan. Fibronectin is therefore an excellent linkage protein between these different molecules to stabilize and strengthen the ECM.

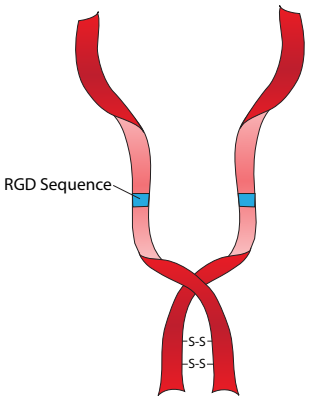


Figure 5. Fibronectin. The C-terminus of each subunit is at the bottom of the figure. The RGD sequence is a binding site for integrin receptors.

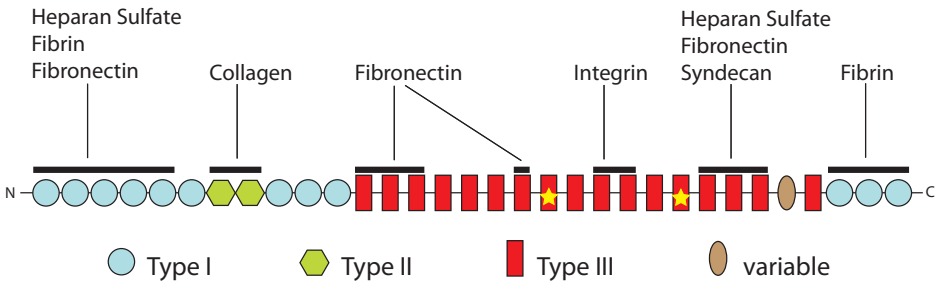


Figure 6. Each fibronectin subunit is composed of about 30 modular domains, of which there are three major structural types. Binding sites for other molecules are labeled. The two Type III domains that are marked with a star are alternatively spliced domains not found in fibronectin that circulates in the bloodstream, where it helps to promote clotting.

Importantly, in addition to linking a variety of extracellular matrix proteins together, fibronectin also has a site that binds to integrin receptors on cells. Whereas collagen for the most part acts as a passive substrate that cells are willing to attach to or crawl on, fibronectin can *actively* induce cell migration by activation of the integrins. Fibronectin expression along very specific pathways are crucial for the migration of neural crest and other cell types in development. *In vitro* experiments with fibroblasts and other cell types show a marked preference for areas coated with fibronectin over areas coated with collagen. This is also true *in vivo*: an upregulation of fibronectin in response to injury promotes migration of fibroblasts and cells associated with wound healing into the lesioned area.

The integrin binding site is charactized by the presence of an arginine-glycine-aspartic acid (RGD) sequence. If this site is abolished or mutated, the mutant fibronectin does not bind to cells. Similarly, if cells are treated with high concentrations of short peptides that contain the RGD sequence, those peptides bind to the integrins, and the cells ignore fibronectin. Finally, in addition to serving as a linker between other ECM proteins, or even to cells, fibronectin can form fibrils through interaction with other fibronectins.

Laminins

Although there are many other less abundant proteins in the extracellular matrix, laminin is the final ECM molecule to be discussed in this chapter. Laminins are a family of secreted glycoproteins that are found in many ECM formations, and like fibronectin, bind to cells via integrin receptors. The laminin protein is composed of three subunits (α, β, γ) arranged in a cruciform shape. There are multiple isoforms of each subunit

One of the interesting aspects of fibronectin fibril formation is that the self-association site is generally hidden, but is revealed when a cell binds to the integrin-binding site. Thus cell-binding seems to nucleate the formation of fibrils, perhaps helping to form strong anchors in certain situations in which the cell is not migrating, but establishing itself permanently.

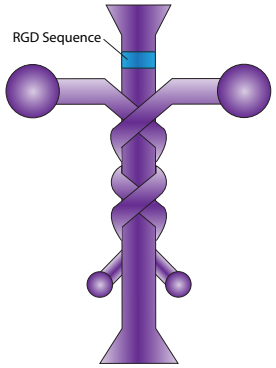


Figure 7. The cruciform structure of laminin is composed of three subunits. The arms bind collagens and sulfolipids, while the foot contains LG domains that bind integrins and carbohydrates.

yielding the variety (15) of laminin proteins catalogued to date. Although laminin contains an RGD sequence like fibronectin, its role in cell adhesion is not universal. Although some cell types have been demonstrated to bind to the RGD site, others clearly bind to other domains of laminin, primarily located on the opposite end of the protein.

Laminin plays a crucial role in neural development, where it acts as a guiding path along which certain axons extend to find their eventual synaptic targets. One prominent example is the retinotectal pathway that leads retinal ganglion cell axons from the eye to the brain. Another example of the role of laminin in development is the guidance of primordial germ cells (PGC). These are cells that eventually become the gametes, but need to migrate from the yolk sac, which is outside the embryo proper, to the site of gonad formation. Laminin is found along this pathway. Interestingly, as the PGCs reached a stretch of laminin very close to the final destination, the adhesion to laminin increased, and this adhesion was found to involve not integrin receptors, but an interaction with a cell surface heparan-sulfate proteoglycan. This and other evidence suggests that migration on laminin may be mediated by integrin receptors, whose adhesivity can be regulated intracellularly, while more static interactions with laminin may be mediated by other types of binding proteins. Finally, as already discussed, laminin is an impotant component of basal lamina, able to form fibrils and networks itself, as well as with collagen IV.

At present, there are 5 α-chain genes, 4 β-chains, and 3 γ-chains known. The α2/β1/γ1 combination is known as laminin-2 or merosin, and is found primarily in the basal lamina of striated muscle. Mutations that affect the function of this laminin cause a form of congenital muscular dystrophy.

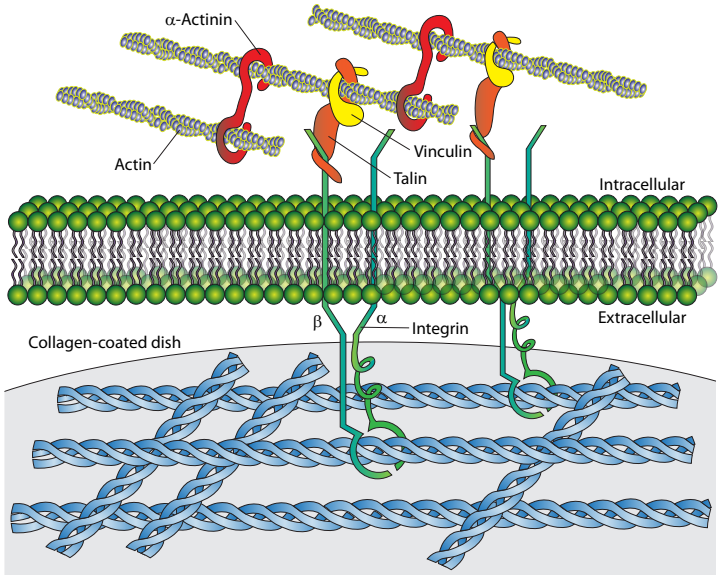
Integrins

The integrins have thus far been introduced as receptors for fibronectin and laminin, but it is a large family with a wide variety of substrates. For example, the focal adhesion (fig. 8) shows an an integrin receptor bound to collagen. As already discussed in the previous chapter, focal adhesions are usually transient, and seen as points of contact as fibroblasts or other migratory cells crawl on a culture dish or slide coated with ECM proteins. In addition to collagen, fibronectins and laminins are also potential binding partners for integrins. As table 1 shows, the diversity of subunits and combinations means that integrins are involved in a wide array of cellular processes, and can bind cell surface proteins as well as ECM. With this variety, it is not surprising that not all integrins bind RGD sequences, although most do. For example, α2β1 integrins prefer

Subunits	Ligand	Distribution
α1β1	mostly collagens, also laminin	widespread
α2β1	mostly collagens, also laminin	widespread
α4β1	fibronectin, VCAM-1	hematopoietic cells
α5β1	fibronectin	fibroblasts
α6β1	laminin	widespread
α6β4	laminin	epithelial cells
αLβ2	ICAM-1, ICAM-2	T-lymphocytes
αIIbβ3	fibronectin, fibrinogen	platelets

Table 1. Integrin receptors, their ligands, and distribution.

Figure 8. A focal adhesion is a dynamic point of contact formed by a cell growing on a collagen-coated dish.



YYGDLR or FYFDLR sequences, and αIIbβ3 binds both the RGD and a KQAGDV sequence strongly. Integrin activation has been shown to initiate signaling pathways, beginning focal adhesion kinase (FAK) or a few other central kinases, which control activities from cytoskeletal rearrangement to cell survival.

Both α and β subunits are transmembrane proteins that pass through the membrane just once. Evolutionarily, they are found only in metazoan species, but they are also found in *all* metazoan species. All integrins but one, α6β4, connect to the actin microfilament cytoskeleton through the β subunit cytoplasmic domain. The α6β4 integrin links to the intermediate filament cytoskeleton, in part because the β4 cytoplasmic domain is very large and extends further into the cytoplasm. On the extracellular side, there is a metal ion coordination site usually occupied by Mg^{++}, that is necessary for ligand binding. There are also several other divalent ion binding sites, The receptor can be found in either an inactive (somewhat bent over towards the membrane) or an active state (straightened up). In the inactive state, the α subunit binds the β subunit closely preventing interaction with the cytoskeleton. However, once a cytoskeletal element such as talin attaches to the β

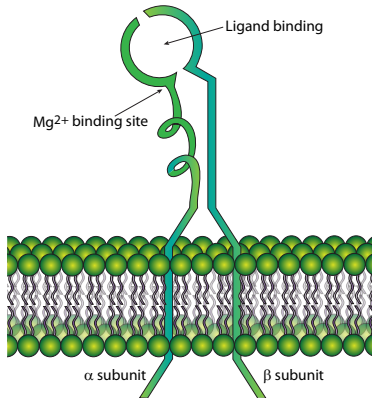


Figure 9. Integrin receptors are composed of two polypeptides that each pass through the membrane once.

subunit cytoplasmic domain, it displaces the α subunit, causing a slight separation of the two subunits and leading to activation of the receptor. In fact, integrins demonstrate what is known as "inside-out" signaling, in which a cellular signal (for example, from the signaling cascade of a growth factor) leads to alterations to the cytoplasmic domain and which shifts the conformation of the extracellular domain to an active straightened-up state in which it can more readily bind to ligands. This is why integrins are so well suited to focal adhesions and other "in motion" adhesions that must adhere and release quickly. Though recycling of receptors also happens, turning them on or off by inside-out signaling is an effective mechanism for fast movement.

As one might expect from an actin-linked structure, focal adhesions and their in vivo equivalents are transient, dynamic points of contact between the cell and the substrate it is crawling over. However, there are many situations in which a cell is not only stationary, it needs to be firmly attached to its substrate in order to gird itself for whatever stressors might come to test its resolve. In these cases, the actin cytoskeleton is too ephemeral for the task.

Hemidesmosomes

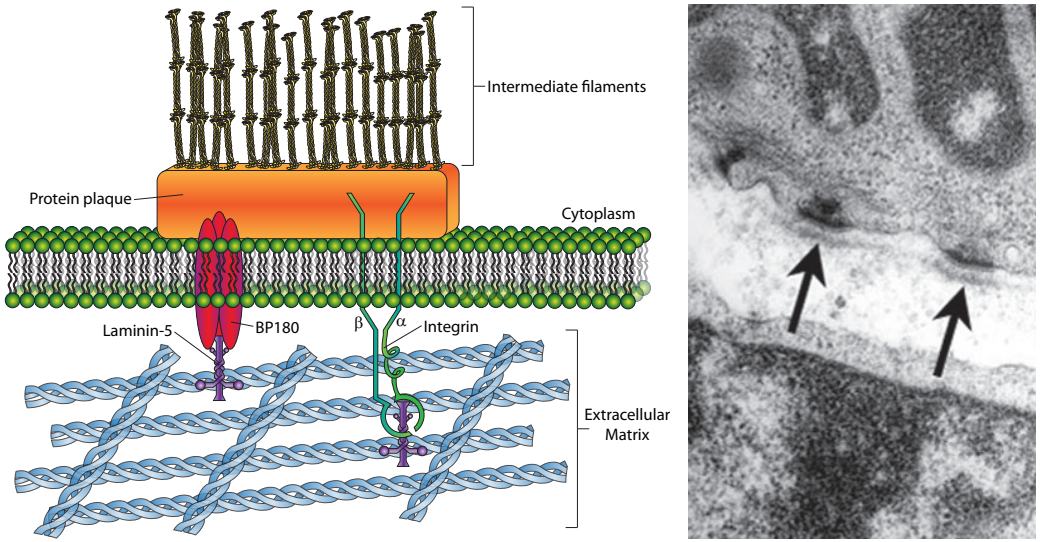


Figure 10. Hemidesmosomes. (Left) Diagram depicting involvement of intermediate filaments and a dense protein plaque reinforcing the membrane at the point of contact. (Right) This shows up as the electron dense areas pointed out by the arrows in this electron micrograph of epithelial cells in mouse trachea. Micrograph released with creative commons attribution license by Nguyen et al, Respiratory Research 7:28 (2006).

Hemidesmosomes, particularly those attaching epithelial cells to their basement membrane, are the tightest adhesive interactions in an animal body. This close contact, and the reinforced structure of these contacts, is crucial for the protective resilience of epithelial layers. Remember the $\alpha 6\beta 4$ integrin? That would be the one that links with intermediate filaments instead of f-actin. Intermediate filaments, as we've already noted, are not dynamic, but about as stable as a cellular component can be. They are also very strong and are used to buttress cellular integrity. So, it is no surprise to see intermediate filaments and the $\alpha 6\beta 4$ integrin playing roles in hemidesmosomes. The distinguishing characteristic of hemidesmosomes though, is the electron-dense plaque. It can be thought of as reinforcement so that when the epithelium is stretched, the cell does not just pop loose leaving behind part of its membrane. The plaque contains several proteins, but the primary component are plectins, the linker proteins that help to bundle intermediate filaments, and connect them to each other as well as other cytoskeletal elements. Another major element of the plaques is BP230, which connects the plaque to keratin. On the extracellular side, in addition to the integrin already mentioned, there is also a transmembrane glycoprotein called BP180, which also binds to laminin elements of the basement membrane.

BP230 and BP180 are named for bullous pemphigoid, the subepidermal bullous disorder characterized by chronic blistering of the skin. It is an autoimmune disorder and in which the aberrant antibody response is to these two hemidesmosomal proteins.

Dystrophin Glycoprotein Complex

Another type of cell-ECM connection is the dystrophin glycoprotein complex (DGC) of skeletal muscle cells. Similar complexes are found in smooth muscle and in some non-muscle tissues. Muscle cells, of course are subject to frequent mechanical stress, and connectivity to the ECM is important in supporting the cell integrity. The DGC uses the large transmembrane glycoprotein, dystroglycan, as its primary binding partner to basal lamina laminin. A sarcoglycan complex and sarcospan are other major transmembrane components of the DGC, but their roles do not appear to include direct interaction with basal lamina. The sarcoglycan complex (consisting of 4 sarcoglycans) is postulated to act as structural reinforcement for the membrane at these contact points. The role of sarcospan, a 4-pass transmembrane protein, has not been demonstrated within the DGC, but homologous proteins in other cells are found in adhesive com-

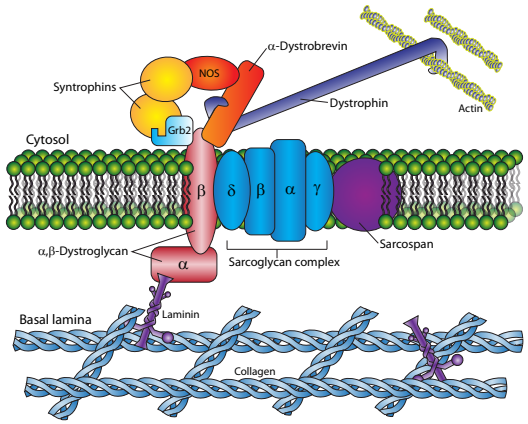


Figure 11. Dystrophin Glycoprotein Complex.

Mutations leading to loss of sarcospan have not been linked to any muscular dystrophies. However, sarcospan is not found in muscular dystrophy patients who have mutations in the sarcoglycans. It thus appears that the tetrameric sarcoglycan complex is required for normal membrane localization of sarcospan.

plexes with integrin receptors, suggesting the same function here. Although the DGCs are long-lived adhesive contact that have more in common with hemidesmosomes than focal contacts, the cytoskeletal component attached to the DGC is actin (via dystrophin), not intermediate filaments. However, it is important to note that the actin cytoskeleton of muscle cells has very different functions from its counterpart in a fibroblast. Mutations to the sarcoglycans, dystroglycan, and dystrophin have all been shown to cause muscular dystrophies.

Desmosomes

Cells will form adhesive interactions with other cells as well as with ECM. Most of these interactions utilize a different set of proteins, although integrins have been found to interact with some cell adhesion proteins. An example of a cell-cell interaction with many similarities to a cell-ECM interaction, but using different adhesion molecules, is the desmosome. Like its basal-lamina-attached couterpart, the hemidesmosome, the desmosome is found in epithelial sheets, and its purpose is to link cells together so that

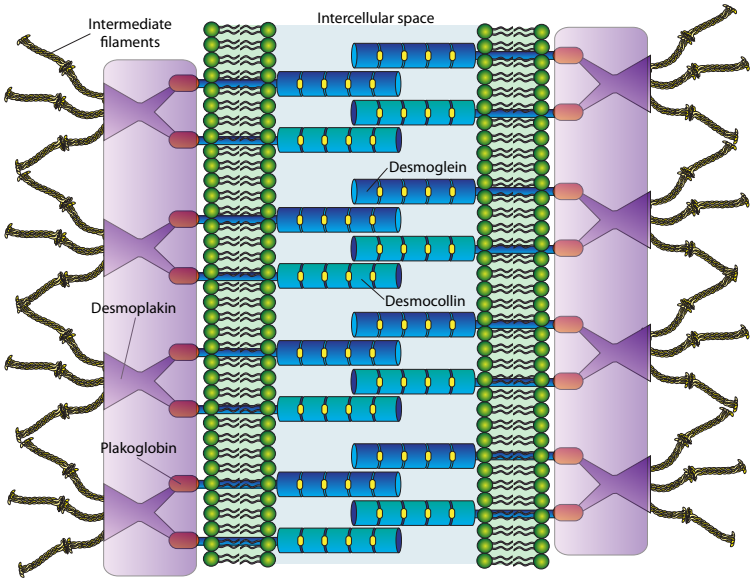


Figure 12. Desmosomes connect the intermediate filaments of adjacent cells across adhesion molecules strengthened by a protein plaque. Desmoglein and desmocollin, the adhesion molecules are members of the cadherin family, and the reinforcing plaque contains plakoglobin, which connects the adhesion molecules to the IF linker, desmoplakin.

pressure is spread across many cells rather than concentrated on one or a few. Desmosomes are necessary for the structural integrity of epithelial layers, and are the most common cell-cell junction in such tissues. The primary structural characteristic of the hemidesmosome, the dense plaque reinforcing the intracellular side of the adhesion, is also found in desmosomes, although it is composed of different proteins. In desmosomes, the plaque is composed primarily of plakoglobins and desmoplakins. The plakoglobins connect the adhesion molecules to the desmoplakins, and the desmoplakins link to intermediate filaments such as keratin.

Another similarity to hemidesmosomes, and one predicted by the involvement of keratin, is the permanence of desmosomes. On the other hand, a key difference between the two types of adhesions is the adhesion proteins involved. The major proteins of the desmosome are desmoglein and desmocollin, both of which are members of the cadherin superfamily of Ca^{++}-dependent adhesion molecules.

Mutations in desmoplakin (on chromosome 6p24) are linked to Carvajal syndrome (also known as dilated cardiomyopathy with woolly hair and keratoderma). Patients are born with woolly hair, and palmoplantar keratoderma appears within the first year. Dilation of the left ventricle and attending weakness in contractility may lead to death from heart failure in teenage years.

Pemphigus vulgaris is another rare disease involving dysfunction of desmosomes. It is an autoimmune disease targeted to the patient's own desmoglein proteins. The reduced epithelial adhesion leads to blistering of skin and mucous membranes.

Cadherins

The cadherin superfamily is comprised of the desmogleins (of which 4 have been identified in humans) and desmocollins (3 in humans), the cadherins (>20), and the protocadherins (~20) as well as other related proteins. They share structural similarity and a dependence on Ca^{++} for adhesive activity, and they can be found in most tissues, and for that matter, most metazoan species. Cadherins are single-transmembrane modular proteins. On the outside of the cell, the cadherin has five domains of similar but not identical structure. It was originally thought that Ca^{++} was used between cadherins to mediate adhesion, but it is now clear that Ca^{++} is bound in between each extracellular domain, apparently coordinating them into a more rigid structure. Cadherins can also act in *cis*, i.e. cadherins from the same cell can form dimers. This property allows a patch of cadherin adhesions such as a desmosome to "zipper" together into very strong clusters.

The cytoplasmic domain of cadherins characteristically binds to a family of proteins called the catenins, and this binding can be regulated by phosphorylation of the cadherin. The most common catenins are α and β, usually with the β-catenin acting as intermediary between cadherin and α-catenin, and the α-catenin linking them to the actin microfilaments. This kind of arrangement is found in both cells that are motile, crawling over other cells that are expressing cadherin, as well as stationary cells. Although this is not the arrangement in desmosomes, the desmosomal plaque protein plakoglobin is a member of the catenin family.

The primary binding site for cadherins appears to be the N-terminal domain (most distal extracellular), although there is evidence that as many as three domains can be involved. Cadherins mostly bind homophilically (E-cadherin binds E-cadherin on another cell, but not P-cadherin), although some cadherins can bind heterophilically (e.g. N-cadherin can bind to either N-cadherin or E-cadherin). Incidentally, these three, E-cadherin (epithelial), N-cadherin (neural), and P-cadherin (placental) are the best-studied cadherins. Both E- and P-cadherins are important in early embryonic development, while N-cadherin has been studied in the context of axon guidance in the developing nervous system. E-cadherin is also a target of scrutiny because it is also important in the metastasis of cancer. In order for a cancer cell to break from the initial tumor, it must downregulate its adhesion to neighboring cells before migrating elsewhere. This is known as the epithelial-mesenchymal transition and is accompanied by decreased E-cadherin expression.

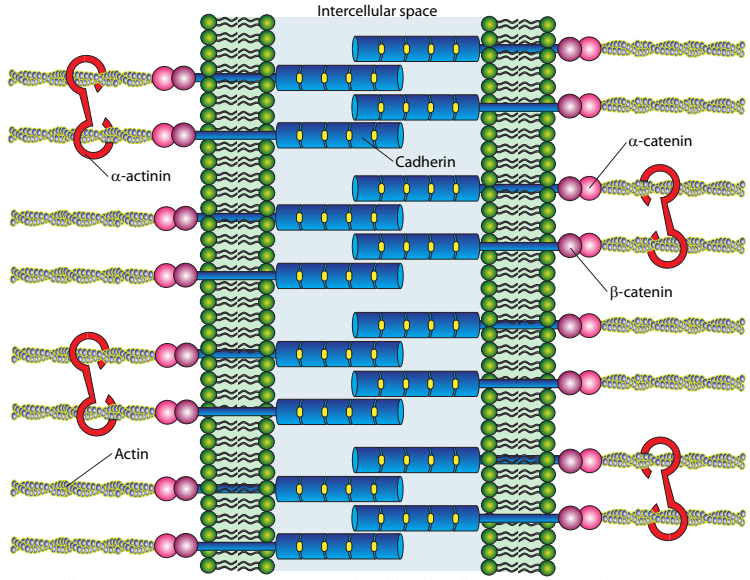


Figure 13. Adherens Junction. This type of cell-cell adhesion is based on interaction of cadherins, which are connected intracellularly to the actin cytoskeleton through the linker proteins α- and β- catenin. Also depicted here is the actin bundling protein α-actinin.

With the patch of cadherin interactions, the adherens junction (fig. 13) looks very similar to the desmosome (fig. 12). Adherens junctions serve some of the same purposes as desmosomes: providing connectivity to neighboring cells, and reinforcing and shaping the cells. However, adherens junctions are mostly localized near the apical surface of epithelial cells, and instead of intermediate filaments, they are connected to actin microfilaments that form a circumferential belt that produce tension and shaping forces in conjunction with myosins that associate with it.

Tight Junctions

Sometimes, holding cells together, even with great strength, is not enough. In epithelia especially, a layer of cells may need to not only hold together but form a complete seal to separate whatever is in contact with the apical side from whatever is in contact with the basal side. That would be a job for The Tight Junction! Well, more accurately, for many tight junctions in an array near the apical surface. Perhaps the best example of the utility of tight junctions is in the digestive tract. The tight junctions that form between cells of the epithelial lining of the gut separate the food and its digestion products from the body at large, forcing macromolecule nutrients to be transported through the epithelial cell by endocytosis/transcytosis to the bloodstream where they can be most efficiently distributed. The tight junctions also form in blood vessels to prevent leakage of blood, and in a variety of organs where liquids must be contained.

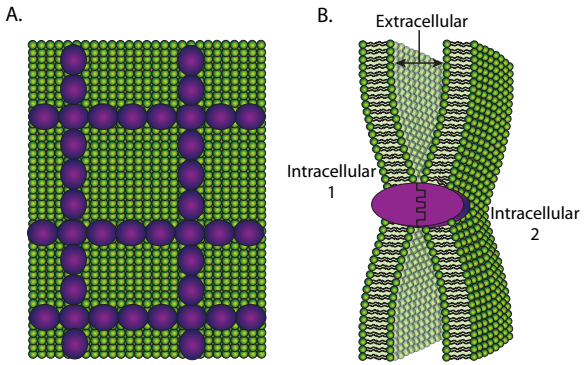


Figure 14. Tight Junctions. (A) Tight junctions are usually present in arrays that seal off one side of an epithelial layer from the other multiple times. (B) Each tight junction is formed by very small transmembrane proteins, claudins and occludins, so that the membranes of opposing cells can come into extremely close contact .

An individual tight junction is formed by the interaction of claudins and occludins. They are each 4-pass transmembrane proteins with both N- and C-termini on the cytoplasmic side; the extracellular side has a very low profile, consisting of one (claudin) or two (occludin) small loops. Because of their small size, when they interact, the membranes are brought together very closely. In order to actually form a seal between cells though, tight junctions must be lined up in close order all the way around the cell, and in fact, usually there are multiple lines, which one could think of as "backup" in case one line develops a leak. Claudin molecules have relatively small cytoplasmic domains and it is not clear whether there are significant interactions with other proteins. However, occludin has a large C-terminal cytoplasmic domain that contains a PDZ-binding domain. PDZ is a protein interaction motif of approximately 80-90 amino acids found in a number of signaling proteins, most often in use to hold signaling complexes near the membrane by interacting with a transmembrane protein, as would be the case here with occludin. These PDZ-containing proteins both have signaling functions and can

act as adapters to the cytoskeleton, primarily the actin filaments. Finally, although an exact mechanism is unclear, elevated levels of Ca^{++}, either extracellularly or perimembranously, is associated with tight junction assembly.

Ig Superfamily CAMs

In addition to occludin and claudins, junction adhesion molecules (JAMs) have recently been found in tight junctions. These molecules are members of a gigantic superfamily of cell adhesion molecules known as the Ig (immunoglobulin domain) superfamily because all of these proteins contain an immunoglobulin loop domain that plays an important part in the adhesion mechanism. The purpose of immunoglobulins (antibodies) is to recognize and adhere to other molecules, so it makes sense that such a structural motif would also be used for other kinds of adhesion. Ig loops fall into two primary categories, again based on the loops of an immunoglobulin molecule. These are the variable (V) loop and the constant (C1) loop. Some IgSF molecules contain a C2 loop, which has amino acid homologhy to the V loop, but structural/size similarity to the C1 loop. The size of IgSF molecules ranges greatly, and the number of Ig loop domains and other domains (e.g. fibronectin Type III domains) can also vary significantly.

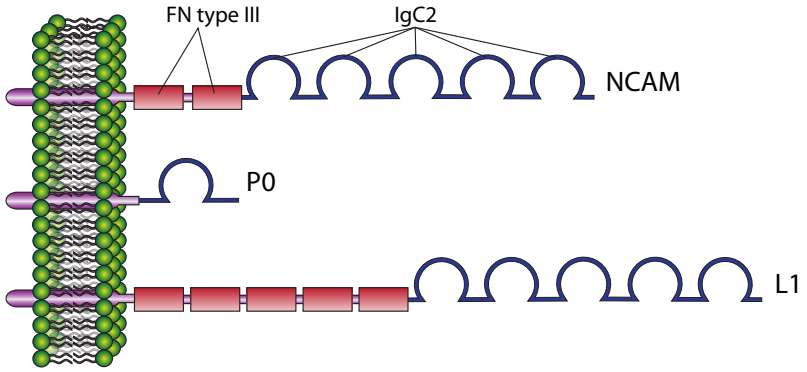


Figure 15. Ig superfamily cell adhesion molecules NCAM, P0, and L1 (top to bottom) are built with fibronectin Type II domains and Ig C2-like domains..

IgSF molecules are involved in a number of cellular processes requiring adhesion. The most obvious, of course, is the immune response in which an immunoglobulin, either secreted or on a cell, binds to a molecule foreign to the body. However, there are many other interactions outside of the immune system that involve IgSF molecules. One well-studied area is in neural development, where IgSF members L1 (L1CAM), NCAM and numerous others are expressed in specific patterns to control the routing of axons as

they make their way from the neuronal cell body to their eventual connections. This path can often be very long, crossing many different cell types, and taking several turns, so a robust guidance system is crucial to make a normal functioning nervous system. Specific combinations of cell adhesion molecules (also called axon guidance molecules in this case) direct these processes even through the extraordinary density of potential (but incorrect) nerve cell targets in the brain. IgSF molecules have been found to bind both homophilically and heterophilically, and for that matter, not just to other IgSF molecules, but to adhesion molecules of other structural families such as integrins.

Selectins

The last major cell adhesion molecule family to discuss is the selectins. Selectins bind heterophilically to oligosaccharide moieties on glycoproteins. In fact the name of the family is based on lectin, a generic term for proteins that bind sugars. The selectins, like cadherins and IgSF molecules are modular glycoproteins that pass through the membrane once. From C-terminus to N-terminus, the selectins are composed of a relatively short cytoplasmic domain, a single transmembrane domain, a series of CR (consensus repeat) or structural domains (from 2 in L-selectin to 9 in P-selectin), an EGF (epidermal growth factor) -like domain, and a lectin-like domain. The lectin-like domain binds a fairly specific oligosaccharide composed of sialic acid, galactose, GlcNAc, and fucose, of which the sialic acid and fucose are most important for recognition. Selectin-mediated adhesion is a Ca^{++} dependent process.

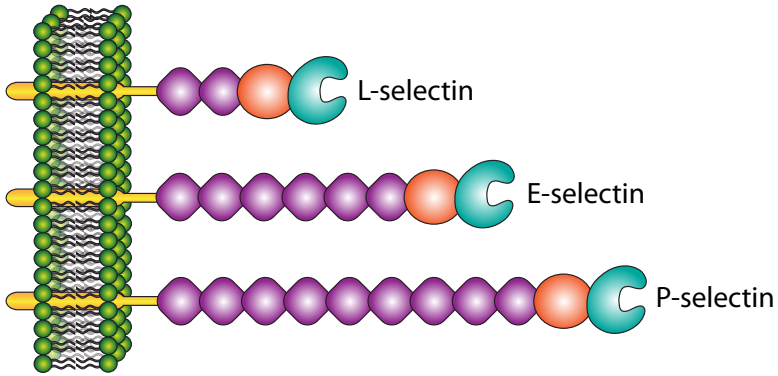


Figure 16. Selectins (L-, E-, and P-) are composed of between 2 and 9 short consensus repeat domains (purple), an EGF-like domain (orange), and the lectin-like binding domain (teal).

L-selectin, which is found on leukocytes, was the first discovered, by virtue of an interesting phenomenon called lymphocyte homing, in which lymphocytes were removed from various peripheral lymph nodes, labeled, and then injected back into the animal.

These lymphocytes would migrate back to the tissues from which they were derived without regard for the site of re-injection. The other two known selectins are E-selectin, which is expressed primarily on endothelial cells, and P-selectin, which is expressed on platelets and endothelial cells. The best-characterized ligand for selectins is PSGL-1, creatively named P-selectin glycoprotein ligand -1. Both E- and P-selectin are an important part of the inflammatory response, mediating the invasion of neutrophils from the bloodstream into the area of injury (fig. 17).

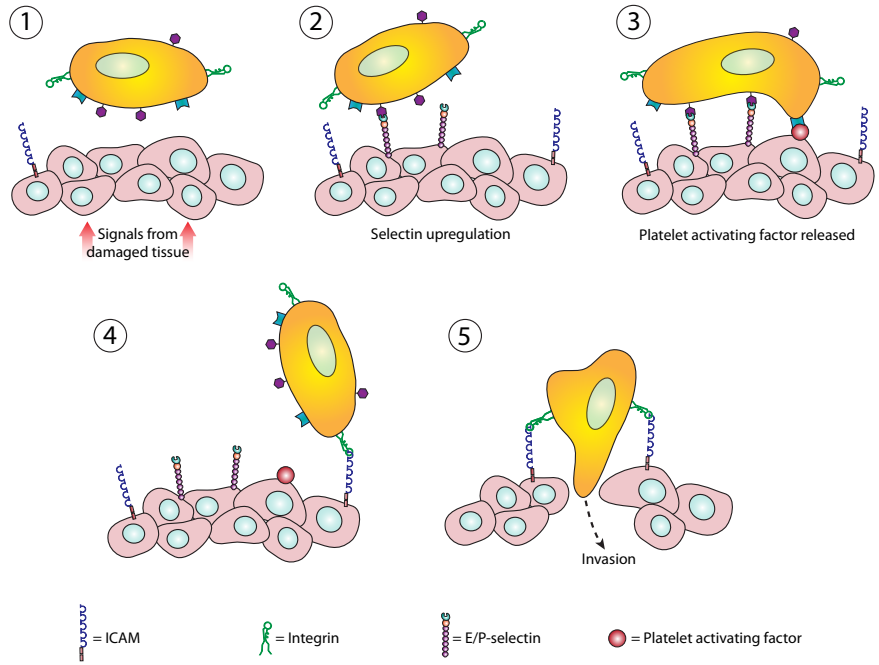


Figure 17. Neutrophil activation and invasion in the inflammatory response. First, endothelial cells in the blood vessel walls are activated by nearby damaged cells. These endothelial cells then begin to express E- and P- selectins, which bind onto neutrophils that are tumbling by in the bloodstream. This interaction slows the neutrophils down so that they are just rolling more slowly in partial contact with the endothelial cells. The endothelials release platelet activating factor, which activates $\alpha 1\beta 2$ and $\alpha M b2$ integrins on neutrophils. Those integrins can bind onto the ICAM (and IgSF molecule) on the endothelial cell surface, stopping their movement. Finally the neutrophil binds to ICAMs on two adjacent cells, then remodels its cytoskeleton as it squeezes between the two to exit the bloodstream and move to the lesion site.

Gap Junctions

Unlike the other types of cell-cell adhesion, the gap junction (sometimes called a nexus) connects not only the outside of two cells, it connects their cytoplasm as well. Each cell has a connexon (aka hemichannel) made of six connexin proteins. The connexins may be all of the same type, or combinations of different ones, of which there are 20 known in humans and mice. The connexon interacts with a connexon on an adjacent cell to connect the cytoplasm of both cells in a gap junction.

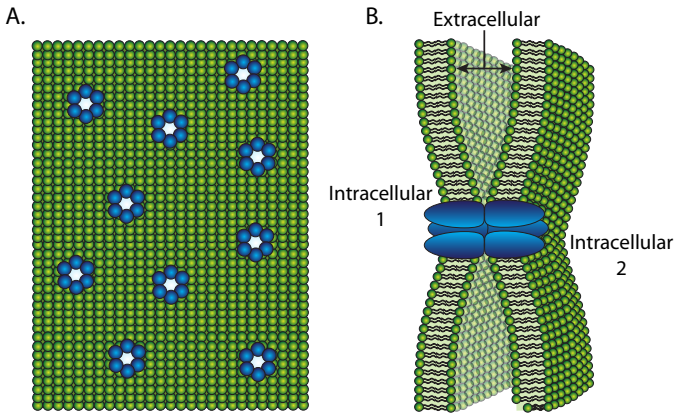


Figure 18. Gap Junctions.

The gap junction pore size varies depending on the type of connexins, but generally, the molecules under 1 kDa are able to pass through while larger ones can not. Therefore, cells connected by gap junctions are electrically connected (ions can freely pass), they can share cellular energy (ATP), and second messenger signaling molecules like Ca^{++} or IP_3, but not most proteins or nucleic acids. The pores are not always open, but are controlled by phosphorylation of several serines in the intracellular domains of each connexin.

Although they have now been found in most metazoan tissue types, they are particularly important in heart muscle. Here, the gap junctions insure efficient propagation of contractile signals so that the cardiac muscle can contract in synchrony. It is also important in cardiac development: gene knockout of connexin43, the primary heart connexin, leads to delayed looping of the ascending limb of the embryonic heart tube, which means malformations especially in the right ventricle, tricuspid valve, and subpulmonary outflow tract.

When most people, including most biologists, think about neuronal connections and synapses, they think of chemical synapses in which one cell signals to another by release of neurotransmitters. However, it is now well established that in the CNS, electrical synapses through gap junctions are a significant part of the repertoire of neural communication. The retina is an excellent example with numerous gap junctions between neurons. In fact, light-activated neurotransmitters can activate protein kinase pathways that phosphorylate connexins, thus altering conductance through the gap junctions. A striking example is the gap junction-based electrical coupling of cone photoreceptor neurons. They are coupled near the base of the cells, so that excitation of one drives excitation of several others. This is important in generating a clear visual signal because the primary reaction, phototransduction, is a dirty process. Due to the simple presence of random photons bouncing about, the signal to noise ratio of light-induced excitation is very low. However, because electrical coupling sums up the signal of near neighbors but not the background noise, the signal output from these neurons has an improvement in signal to noise ratio of ~77%! This topic is reviewed in Bloomfield and Volgyi, *Nature Reviews (Neuroscience)*, **10**:495-506, 2009.

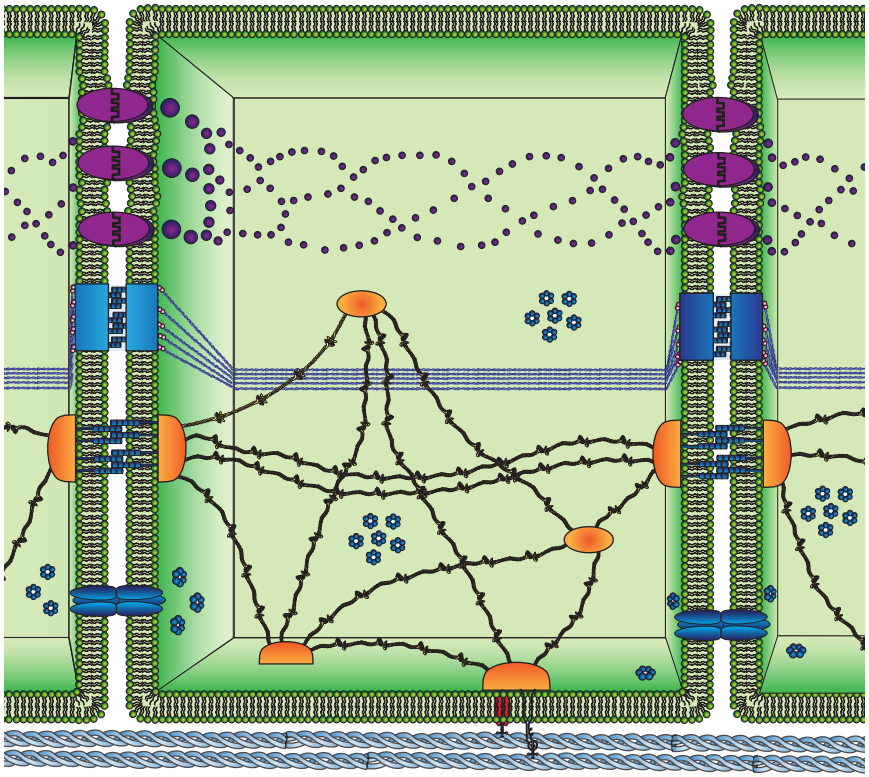


Figure 19. An overview of cellular adhesions. From top to bottom, there are tight junctions (purple), adherens junctions with f-actin (blue), desmosomes (orange) connected to intermediate filaments, and gap junctions (blue). There are also hemidesmosomes (orange) on the basal surface attached to the basement membrane.

Cell Communication :

Signal Transduction

Using this book: This book is designed to be used in both introductory and advanced cell biology courses. The primary text is generally on the left side of the vertical divider, and printed in black. Details that are usually left to an advanced course are printed in blue and found on the right side of the divider. Finally, additional biomedically relevant information can be found in red print on either side of the divider.

Metazoan organisms are not just conglomerations of cells that happen to stick together. The cells each have specific functions that must be coordinated with one another in order to assure the survival of the organism and thus the shared survival of the component cells. If coordination is required, then a method of communication between cells is also required. In fact, it is even more complicated than that because the communications between the cells only scratches the surface (yes, bad pun intended) and the intracellular communication that goes on to coordinate multiple cellular activities in response to an external signal is usually far more complex than the initial transmission of that signal.

There are three primary modes of intercellular communication. These are (1) direct contact between signaling molecules bound to the membranes of two adjacent cells, (2) short-range soluble signals that diffuse over short distances, and (3) long-range soluble signals that are secreted into the circulation to be carried anywhere in the body.

An example of juxtacrine signaling is exemplified by the activity of some cell adhesion or ECM proteins, such as laminin, that do not just allow a cell to move over them, but act as signals to promote increased motility. This likely happens by activation of integrin receptors on the moving cell, which then initiate and coordinate changes through the rest of the cell to accomplish the change in activity. Another example is the Delta-Notch pathway used in embryonic patterning. Delta, a

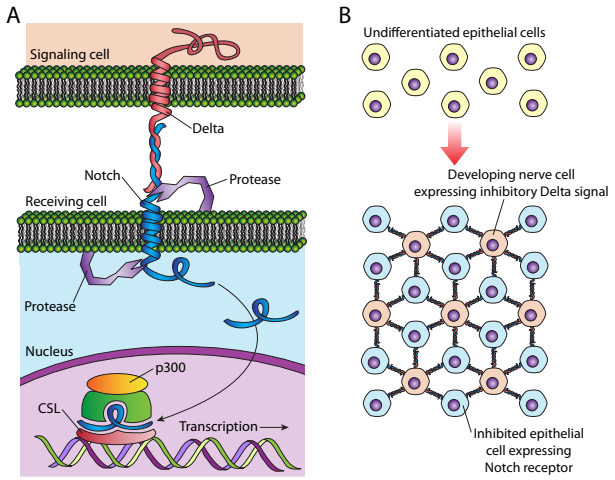


Figure 1. Delta-Notch signaling.

transmembrane protein on the signaling cell, binds to Notch, a receptor on the receiving cell. Notch alters its conformation, allowing its cytoplasmic domain to be cut off by γ-secretase. The cytoplasmic domain then translocates into the nucleus, where it acts as an activating transcription factor by binding with CSL. In the example sketched in fig. 1B, stochastic upregulation of delta in one cell activates notch in the surrounding cells, which then activates a specific differentiation pathway for them. Thus the central cell may be a sensory neuron, like a hair cell, while those immediately surrounding it are support cells like glia. This type of signaling imposes a spacing pattern on the expression of neurons (or other cell).

Diffusion limited signals from near neighbors is called *paracrine* signaling, and sometimes the signals can act on receptors right on the cell that secreted the signal, which would be *autocrine* signaling. Paracrine signals are only active if they can bind to a cell above a critical concentration to activate a signaling pathway. Therefore, as the signals diffuse away from the source, there is a cutoff, beyond which the concentration of signal is insufficient to activate a receiving cell. Growth factors are often paracrine signals. Although they do often encourage growth, they are also often survival factors. In that context, Nerve Growth Factor (NGF) is secreted by target cells that then reward the neurons that make the right connections by providing NGF for their survival. Those neurons that head off in the wrong direction, are unable to obtain NGF, and they do not survive, promoting efficiency and a better signal:noise ratio within the nervous system.

Endocrine signaling is essentially whole-body signaling. A signal produced by a hormone-producing gland is secreted into the bloodstream, where it becomes accessible to nearly any cell in the body. Of course, not every cell will respond to the hormone: like every other case of intercellular signaling, response is wholly dependent on receptors, so only the cells that have receptors to recognize the signal will react. For example, estrogen is released into the circulation, but in females, only some organs show significant impact when estrogen levels are significantly altered. Most tissues are unaffected. Endocrine signals may circulate in other extracellular fluids such as lymph.

Receptors and Ligands

A protein that happens to bind something is not necessarily a receptor. A receptor is defined as a protein that binds to an extracellular ligand, and then undergoes a conformational or biochemical shift in such a way that it initiates a chain of intracellular events by which the cell reacts to the extracellular signal. What are these ligands and their receptors?

The Delta-Notch pathway is well characterized and somewhat more complicated than portrayed in the paragraph to the left. The cleavage of Notch involves two proteases and two sites. Once the Notch cytoplasmic domain binds to CSL, it displaces a number of co-repressors bound to the CSL, and also recruits MAM (Mastermind-1) as a coactivator. MAM recruits histone acetylases to allow further increase transcription of targeted genes, but also recruits kinases that initiate the process of targeting the Notch cytoplasmic domain for ubiquitin-mediated destruction. The expression of Notch-controlled genes is thus self-regulated and shuts off soon after Delta is no longer available. Reviewed in R.A. Kovall, *Curr. Opin. Struct. Biol.* **17**: 117-27, 2007.

Intercellular signals span a very wide range of molecule types. Some are simple gases, like NO, while others are amino acids or derivatives, including glutamate, dopamine, or epinephrine. Lipids such as steroids (e.g. estrogen, cortisol) or eicosanoids (e.g. prostaglandins, leukotrienes) can be intercellular messengers. Finally many signals are peptides or even complex proteins (recall our juxtacrine signaling example). Although most are recognized by cell surface receptors, this is not always the case since, for example, steroids are lipid-soluble and can diffuse through the plasma membrane.

Receptors are a far less varied group of molecules, since they are all proteins, though it must be said that they represent many different protein structures and functions. In general, receptors are very specific for their ligands, but the specificity is not mutual: ligands can be rather promiscuous and bind with multiple receptors. This is part of the coordination aspect of signaling, though as a single ligand can initiate different effects in different cells depending on what receptor is expressed. The remainder of this chapter will delve into some of the intracellular signaling cascades that are characteristic of particular receptor types.

Because receptors, even at high density, represent only a minute fraction of the surface area of the cell, and therefore an even tinier fraction of the volume of the cell, the activation of a receptor must be amplified in order for it to initiate cellular activities (e.g. locomotion, growth, cell cycle progression). Thus one of the first things a receptor does upon activation is to initiate a signaling cascade. This aptly named sequence of events begins with the receptor activating an enzyme. The enzyme may be the cytoplasmic domain of the receptor itself, or it may be an independent protein but closely linked to the receptor. The enzyme does what enzymes do: it rapidly converts substrate molecules into product molecules. In this case, sometimes the product is an activator for another enzyme, and sometimes, the substrate is an inactive enzyme and the product is an activated enzyme. Either way, because of the high activity rates, the single activation of the receptor has increased first to tens or hundreds of enzyme activations, and each of those activates hundreds, and so on, so that the effect of the receptor can be rapidly distributed throughout the cell.

7-TM receptors (G-protein-coupled)

The 7-transmembrane receptors, or G-protein-coupled receptors are, unsurprisingly, a family of proteins that pass through the cell membrane 7 times. The amino terminal is extracellular and the carboxyl terminal is intracellular. Figure 2 shows the transmembrane regions spread out for clarity, but the transmembrane domains actually

form together in more of a cylindrical shape. 7-TM proteins are used as receptors for neurotransmitters such as epinephrine (β-adrenergic receptor), acetylcholine (muscarinic receptor), and serotonin, as well as hormones like glucagon or thyroid-stimulating hormone, and even non-molecular ligands such as light! Rhodopsins are a class of 7-TM receptors that are activated when they absorb a photon (fig. 5). Activating this family of receptors, whether by photon or by more conventional ligand binding, induces a conformational change in the cytoplasmic domain that alters the interaction between the receptor and a protein complex known as a heterotrimeric G protein.

The heterotrimeric G protein consists of an α, β, and γ subunit, of which the α subunit can bind either GTP or GDP, and can hydrolyze GTP to GDP. When the 7-TM receptor is inactive, the G protein complex is usually nearby associated with the membrane by myristoylation or palmitoylation of the α subunit and farnesylation or geranylgeranylation of the γ subunit. Once the 7-TM receptor is activated, it associates with the heterotrimeric G-protein, which causes the G_α to let go of the GDP and bind to a GTP. This then dissociates the G_α from the other two subunits. It can then associate with and activate an enzyme to expand the signaling cascade. One of the two classical pathways starts with G_α activation of adenylate (adenylyl) cyclase. Adenylate cyclase (AC) converts ATP to cAMP. Since ATP is plentiful and AC is a relatively fast enzyme, the first amplification of the signal comes with generation of the "second messenger" molecule

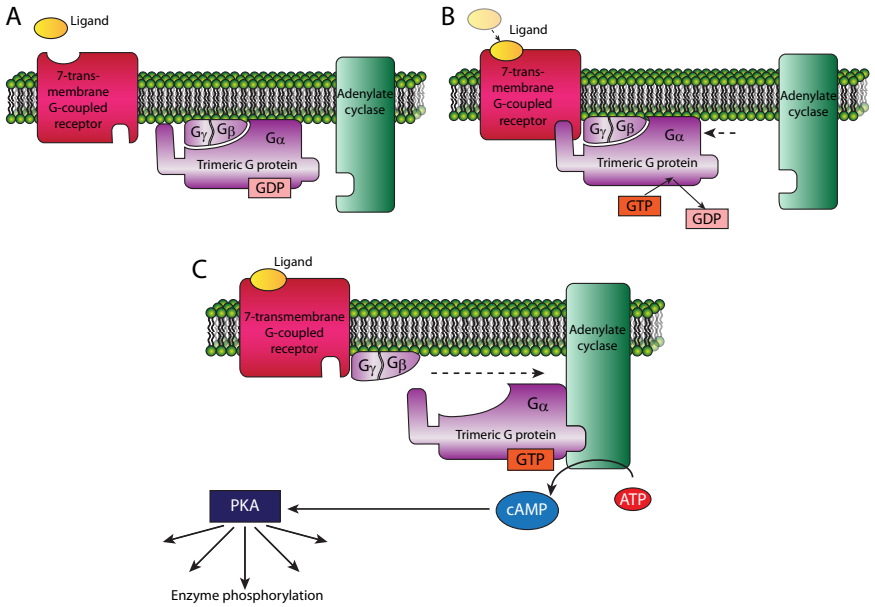


Figure 3. The heterotrimeric G-protein can act as a timed activator of adenylate cyclase.

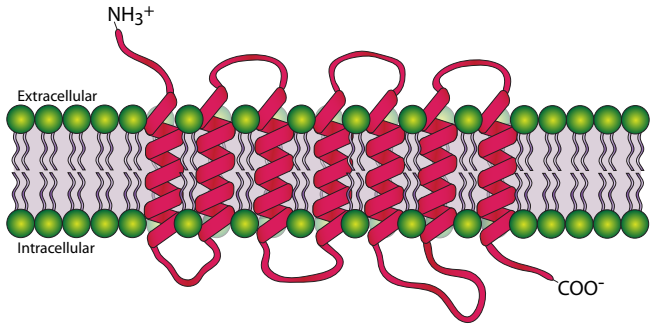


Figure 2. 7-TM receptor.

cAMP. Each cAMP molecule can then activate other enzymes, the primary one being protein kinase A. PKA can then phosphorylate a variety of substrates to alter cellular activity by gene expression, molecular motors, or metabolic changes.

The other classical pathway for 7-TM receptors is the activation of phosphlipase Cβ, also by Gα. PLCβ actually produces two second messenger molecules: it hydrolyzes phosphatidylinositol into diacylgylcerol (DAG) and inositol triphosphate (IP_3). IP_3 primarily induces the release of Ca^{++} from the endoplasmic reticulum. The DAG can activate protein kinase C. PKC is also activated by Ca^{++} and both Ca^{++} and DAG can activate PKC synergistically. Protein kinase C is an important central kinase that has been shown to phosphorylate and control the activity of numerous other enzymes from cytoskeletal elements to transcription factors.

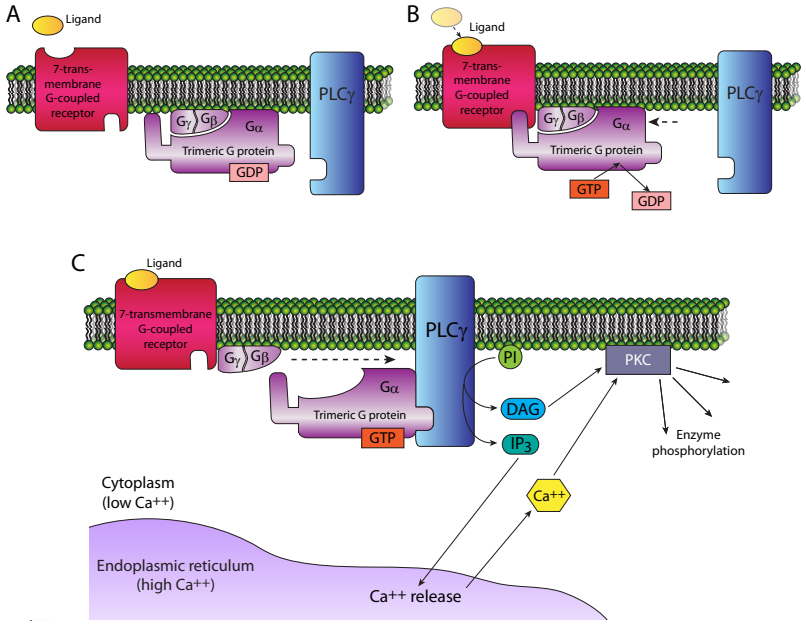


Figure 4. G-protein activated Phospholipase C. (A) The G-protein is inactive with GDP bound. (B) Upon ligand binding, G-protein binds receptor, exchanges GDP for GTP, and G_α dissociates. (C) Gα-GTP activates PLCγ, whic produces DAG and IP_3. The latter induces Ca^{++} release into the cytoplasm and together with DAG, activates PKC.

Second messengers must have two properties. They must be small enough to diffuse effectively, and the cell must be able to generate them quickly. Ca^{++} and cAMP fall into this category. Furthermore they can both be removed from circulation fairly quickly: the former by Ca^{++} pumps in the ER and Golgi, and the latter by phosphodiesterase activity. When the G-protein-pathway was discovered, the use of lipid second messengers

An interesting variation from the classic 7-TM pathways starts with the rhodopsin receptors in rod cells. These receptors bind photons for activation, and engage a heterotrimeric G protein. The Gα-GTP then binds to the g subunit of phosphodiesterase (PDE), activating it and catalyzing conversion of cGMP to GMP. As cGMP decreases, ion channels close, polarizing the membrane and changing the signal from the rod cell to the brain (via connecting neurons).

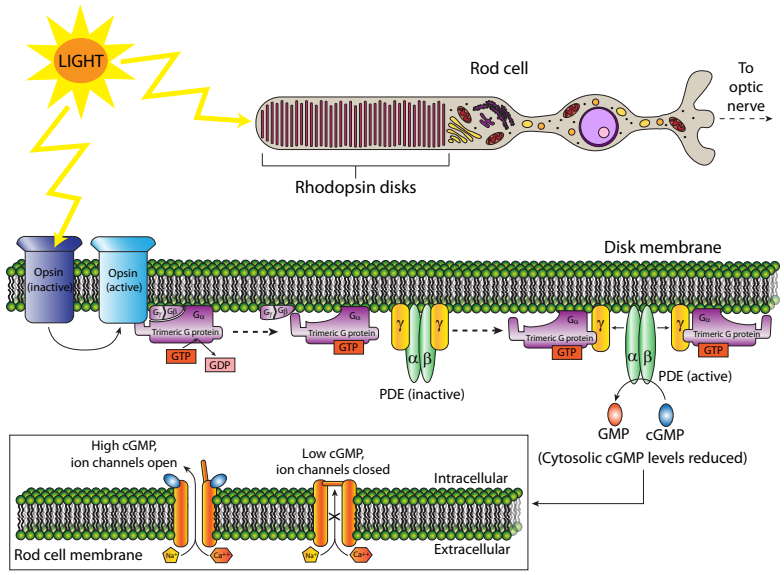


Figure 5. Activation of the 7-TM receptor rhodopsin by light.

was surprising. Membrane phospholipids were largely ignored at the time as simple static components of membranes. It is now clear that some of the phospholipids are biochemically active, with several enzymes that modify them, including phospholipases, phospholipid kinases, and phospholipid phosphatases. Some of these enzymes have a variety of functions because their substrate of product may be an important messenger molecule. For example, PI3K (phosphatidylinositol-3-kinase) is a central signaling kinase because its product, PIP3 (phosphatidylinositol (3,4,5)-triphosphate) is an activator for Akt/PKB and other enzymes that can activate several signaling pathways.

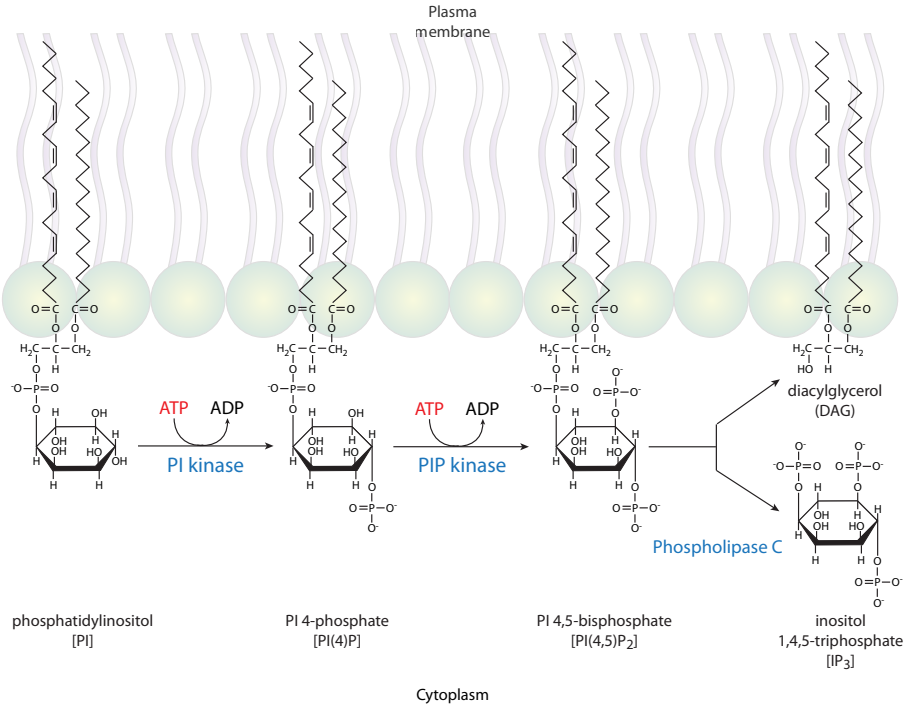


Figure 6. Modifications of Phosphatidylinositol generate various biologically active species.

The activation must eventually end, and it does so when G_α hydrolyzes the GTP bound to it. In this way, the G_α acts as a kind of timer for the signaling cascade. This is important because for signaling to be effective, it must be tightly controlled. Very early in this course, it was pointed out that Ca^{++} is kept at a very low concentration in the cytoplasm of the cell because we want Ca^{++}-sensitive mechanisms to be able to react quickly to an influx of calcium, but we equally want to be able to quickly turn off the signal as needed, and that is obviously much easier to do by sequestering a small amount of Ca^{++} than a lot of it. Similarly, if sustained activity of a recipient cell is called for, it is accomplished by continuous activation of the receptor and not by a long-lasting effect

from a single activation. This ensures that if a cellular effect must be abruptly and quickly cut off, it can be accomplished without a significant lag period between cessation of hormone secretion and cessation of intracellular signaling.

The receptor is a part of another shutoff mechanism as well: to prevent overstimulation, the receptors are desensitized for a short time after they have activated. G-protein-coupled receptor kinase (GRK) phosphorylates the 7-TM receptor. The phosphorylation creates recognition sites for arrestins. The arrestins have a variety of functions, the simplest of which is to act as a competitive inhibitor of G-protein binding by the receptor. This is a relatively short-lived desensitization.

For longer desensitization, arrestin binds to AP2 and clathrin, nucleating formation of a clathrin-coated endocytic vesicle. This removes the receptor from the cell surface, desensitizing the cell for a much longer period of time than simple competition between arrestin and G-protein.

The arrestins have another potential function. They can act as scaffolding proteins that bring a completely different signaling complex to the 7-TM receptor. Figure 8 shows an example in which the 7-TM receptor is used to activate a Jun transcription factor.

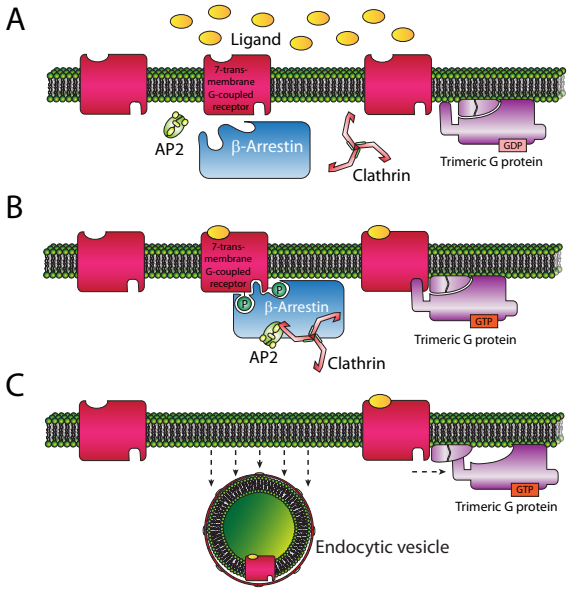


Figure 7. (A) After GRK phosphorylates a 7-TM receptor, arrestin can bind. In some cases, arresting can also bind AP1 and clathrin, (B) nucleating the formation of a clathrin-coated vesicle and endocytosis of the receptor (C).

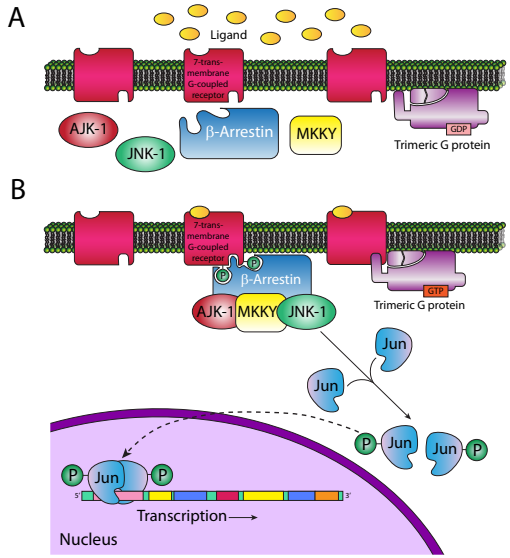


Figure 8. Interestingly, arrestins can also act to initiate a completely different type of signaling cascade from a 7-TM receptor. Here, the Jun transcription factor is activated.

The β-arrestin brings AJK-1, MKKY, and JNK-1 to activate JNK-1, which can then phosphorylate Jun. This allows translocation of phospho-Jun into the nucleus and subsequent dimerization, converting it into an active transcription factor.

What are some of the cellular actions that can be evoked by 7-TM receptor activation? Ca^{++} dynamics will be addressed in a separate section. IP_3 has been shown to evoke contraction of smooth and skeletal muscle, actin polymerization and cell shape changes, calcium release from intracellular stores, opening of potassium channels, and membrane depolarization. cAMP has been implicated in control of glycogen breakdown and gluconeogenesis, triacyglycerol metabolism, secretion of estrogens by ovarian cells, secretion of glucocorticoids, and increased permeability of kidney cells to water.

Receptor Tyrosine Kinases

In contrast to the 7-TM receptors, the receptor tyrosine kinases (RTK) pass through the membrane only once, and have a built-in enzyme domain - a protein tyrosine kinase. RTKs must dimerize to be functional receptors, although individual RTKs can bind to their ligands. The ligands also dimerize, and when a dimerized receptor is activated, the kinase domains cross-phosphorylate the cytoplasmic domain on the other receptor

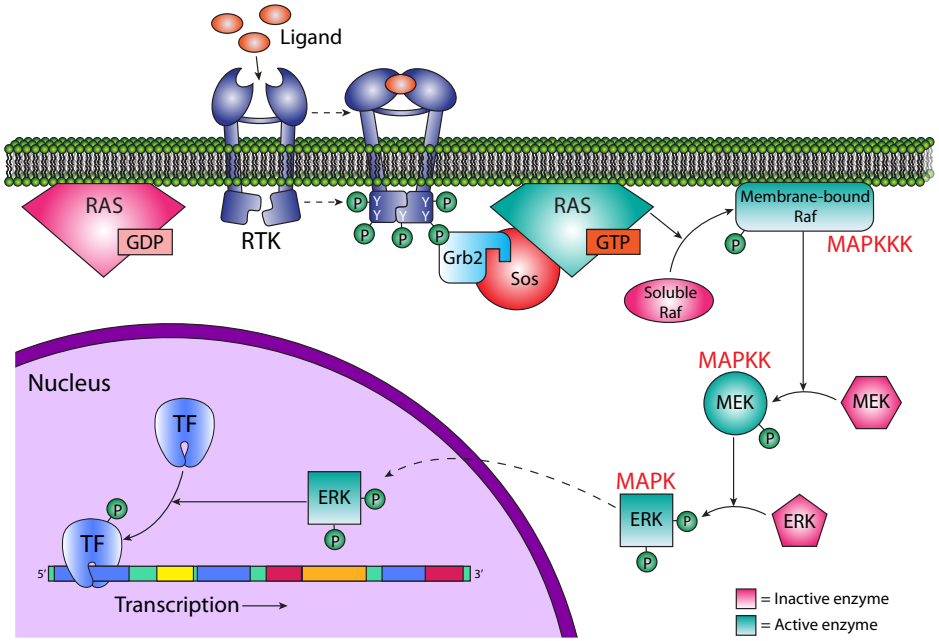


Figure 9. Receptor Tyrosine Kinases can activate the MAP pathway.

unit. This phosphorylation is necessary to form recognition sites for scaffolding or effector proteins. Figure 9 shows an example of an adapter protein, Grb2, which binds to a phosphorylated SH2/SH3 type domain on the receptor as well as to Sos (a guanine nucelotide exchange factor), which binds to and activates the GTPase Ras by exchanging a GDP for a GTP. This is the start of a very common RTK intracellular signaling pathway, the MAP kinase pathway. Following activation of Ras, it can activate Raf by phosphorylation and translocating it from the cytoplasm to the inner surface of the plasma membrane. Raf is a Ser/Thr kinase (also known by the unwieldy but fun to say, MAP kinase kinase kinase) that phosphorylates MEK (aka MAP kinase kinase). MEK is interesting because it is a dual-specificity kinase, phosphorylating both Ser/Thr sites as well as Tyr sites. The targets we are particularly interested here though, are MAP kinases (mitogen activated protein kinase), also known as ERKs (extracellular signal regulated kinases).

Each kinase along the canonical MAP kinase pathway has other potential substrates besides the next one in the MAPK sequence, so the variety of cellular responses that can be initiated by this pathway is very broad. There are at least 20 classes of RTK by structural similarity, including the fibroblast growth factor receptor (FGFR) class, epidermal growth factor receptor (EGFR) class, neurotrophin receptor (Trk) class, and insulin receptor class. Some growth factors not only induce growth, but survival, and sometimes proliferation. In fact, mutations to growth factors can be oncogenic (cancer-causing).

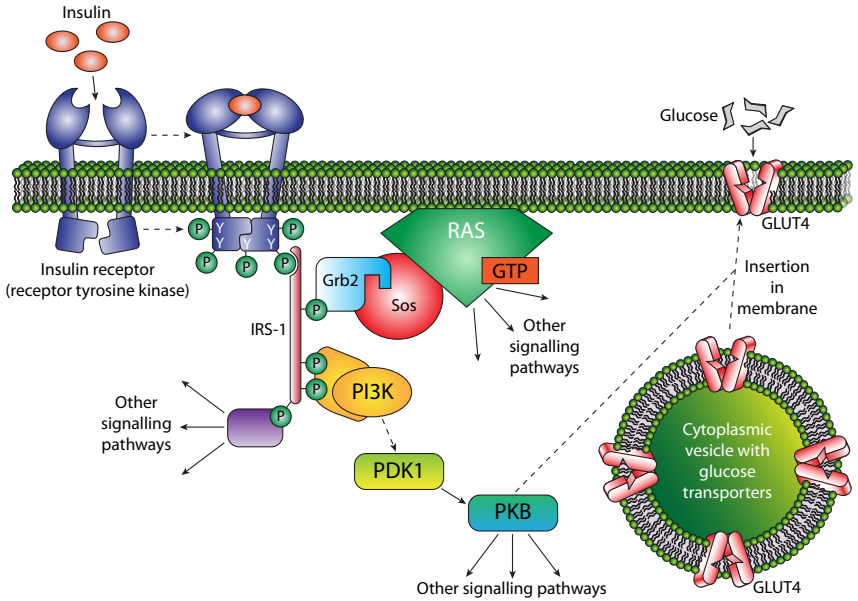


Figure 10. Insulin receptor signaling pathways.

One of the aspects of cell signalling that make studying it both fun and frustrating is the immensity of possibilities. The insulin receptor example above (fig. 10) demonstrates this. When the receptor is activated, the IRS-1 scaffolding protein binds to it, and brings with it binding sites to recruit a number of different signaling molecules such as Grb2-Sos-Ras to head down the MAPK pathway, but also PI3K, which can lead to activation of PDK1 and Protein Kinase B, important in regulation of glucose transport. PKB (also known as Akt), is also an important mediator of cell survival (by inhibiting BAD), cell proliferation, and angiogenesis.

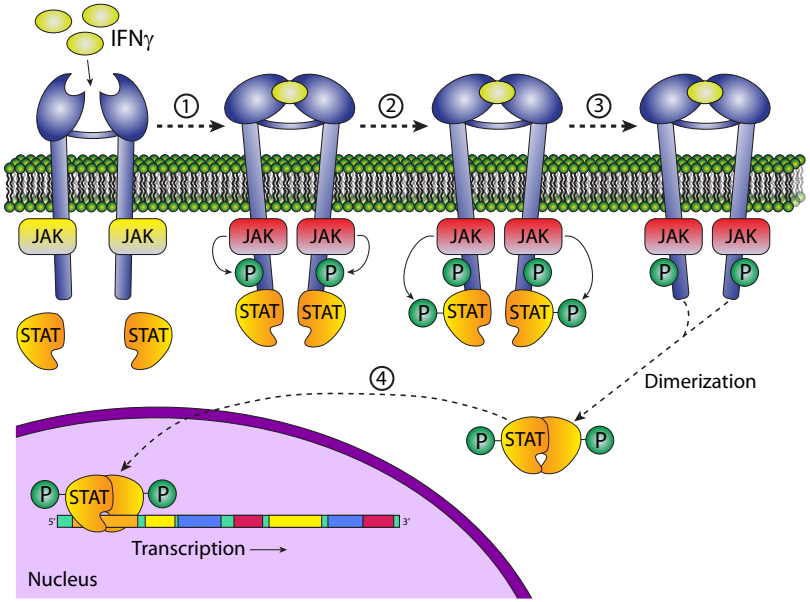


Figure 11. The JAK-STAT pathway.

Activation of cytokine receptors can initiate the JAK-STAT pathway. Cytokines are generally immunomodulatory signals, some of which act as hormones and others in a paracrine fashion. Interferon-γ is an example (fig. 11) of a cytokine, and the inactive RTK receptor binds to JAK (Janus kinase) in the inactive state. Upon ligand binding to the dimerized receptor, the JAK units are activated and the phosphorylate the receptor. This receptor phosphorylation leads to binding of STATs (the creatively named "signal transducers and activators of transcription"), which are then phosphorylated by the still-active JAK. Upon phosphorylation, the STAT-P proteins dissociate from the receptor and dimerize in the cytoplasm, where they are bound by importins and translocated into the nucleus where they act as transcription factors.

Ca^{++} Signaling

Signaling by increasing cytosolic calcium is an important and ubiquitous intracellular coordination mechanism. We already saw that release of Ca++ in muscle cells is required to allow contraction of each sarcomere, and the positioning of the sarcoplasmic reticulum makes possible rapid changes in concentration nearly simultaneous across the entire cell. Another extremely important physiological mechanism that relies on calcium is fertilization. Immediately upon penetration of the egg by the sperm, a wave of intracellular Ca++ increase spreads across the egg starting from the point of fertilization. This activates CaMKII (a kinase) and calcineurin (a phosphatase). Both are needed to overcome meiotic arrest and may also be necessary in initial embryonic development by control of chromatin decondensation, nuclear-envelope formation, and the movement and fusion of the two nuclei.

The aforementioned CaMKII is Ca++/calmodulin-dependent kinase II, and illustrates a fairly common theme, which is the use of Ca^{++}-binding proteins as intermediate Ca++ sensing activators. Calmodulin is a ubiquitous calcium-binding protein in eukaryotes and its importance is highlighted by the extraordinarily high homology across species. In normal cytosolic Ca^{++} levels, the relatively low affinity of the 4 Ca^{++} binding sites on calmodulin are unfilled. But when Ca^{++} concentrations rise, they occupy the sites, causing a conformational change in calmodulin and allowing it to interact with other proteins. In addition to calmodulin, troponin-C, and PKC, a few other important calcium-sensitive proteins are calsequestrin, a Ca^{++} buffer protein, gelsolin, the f-actin severing enzyme, the protease calpain, and calretinin, an activator of guanylyl cyclase (which makes the second messenger cGMP).

Guanylate (guanylyl) cyclase is also an important player in signal transduction by nitric oxide (NO). Nitric oxide is a gas produced by the action of nitric oxide synthase (NOS) on the substrate amino acid arginine. It is used as a super-soluble signal that passes through cells easily. However, it requires relatively high concentrations for physiological effect, so it is strictly a paracrine factor working on near neighbors. Perhaps the best studied example of NO signaling is vasodilation, in which the NOS-expressing endothelial cells of a blood vessel release NO to the smooth muscle cells surrounding them. The NO binds to and stimulates guanylate cyclase. The resulting increase in cGMP concentration leads to relaxation through multiple targets of protein kinase G.

Finally, no discussion of signal transduction would be complete without at least a fleeting mention of the extraordinary crosstalk (fig. 12) that can occur between the different pathways mentioned.

Sildenafil (Viagra) and its chemical siblings take advantage of this pathway by inhibiting cGMP-specific phosphodiesterases (PDE5) which normally break down cGMP to limit the response to NO. However, it should be noted that though PDE5 expression is limited, it is expressed not only in the genitalia but in the retina as well.

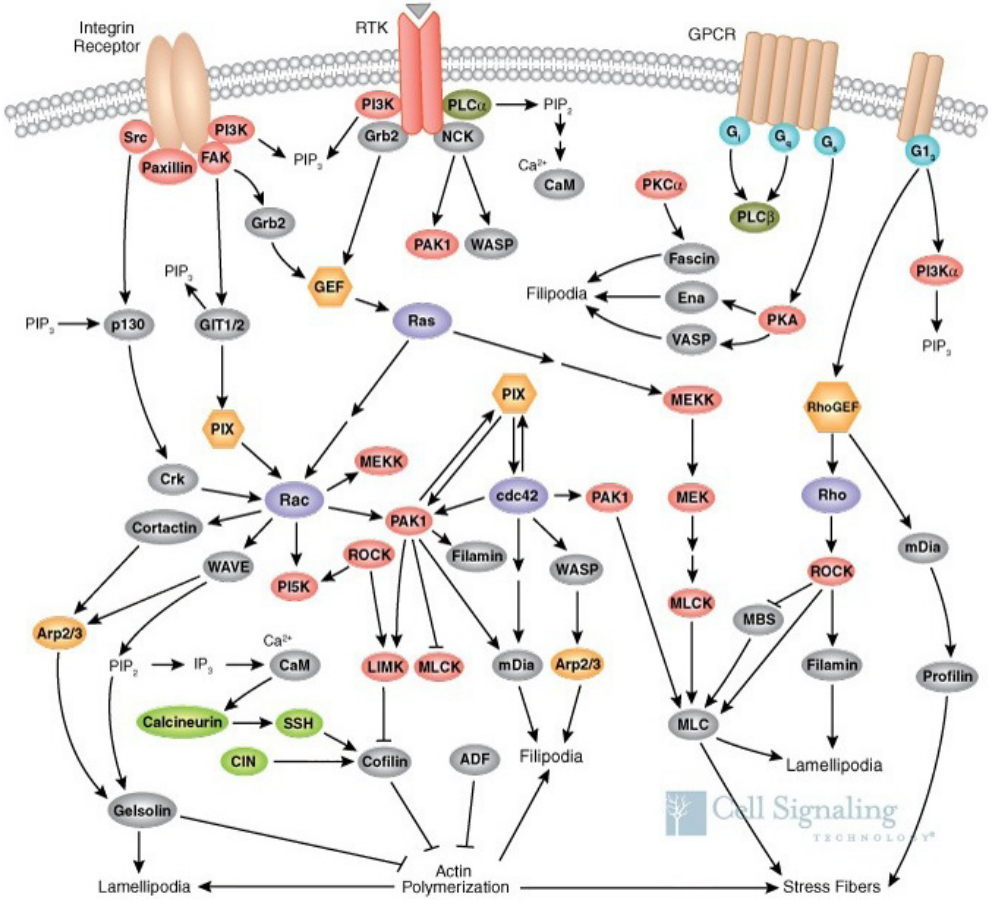


Figure 12. Signal transduction in actin dynamics. This figure comes from Cell Signaling Technology, Inc.

The figure represents only one small part of the signaling that happens inside a moving cell. Not only are some parts of the cell forming filopodia to help determine where to go, other parts or ruffling up the lamellipodia, and still others inducing motor proteins to rearrange the cytoskeleton in the proper way to facilitate bulk transport internally even as the leading edge of the cell is thrusting forward to make contacts externally. All of this must be coordinated by crosstalk between signaling systems as depicted, not to mention signaling related to metabolism, or gene expression, or even cell cycle, all of which are happening simultaneously inside the cell.

Cell Cycle :

Life cycle of the cell and Gametogenesis

Using this book: This book is designed to be used in both introductory and advanced cell biology courses. The primary text is generally on the left side of the vertical divider, and printed in black. Details that are usually left to an advanced course are printed in blue and found on the right side of the divider. Finally, additional biomedically relevant information can be found in red print on either side of the divider.

The Prokaryotic Cell Cycle

Cells, whether prokaryotic or eukaryotic, eventually reproduce or die. For prokaryotes, the mechanism of reproduction is relatively simple, since there are no internal organelles. The process consists of three distinct but short phases: first, a growth phase in which the mass of the cell is increased, then the chromosomal replication phase, and finally the chromosomes are separated and the cells are physically split into two independent new cells. In bacteria, these are referred to as the B, C, and D periods, respectively. Initiation of the reproductive process appears to be primarily a function of cell size. The length of the overall cell cycle is determined by the B period, as the C and D periods have relatively fixed time constraints. The length of B is determined, in part, by environmental conditions and the gain in cell mass. Generation times for bacteria can vary from under half an hour to several days, although most bacterial cultures in laboratory settings and nutrient-rich media have generation times under a day.

DNA replication has already been covered in detail in chapter 7. In bacteria, the process is initiated at the origin of replication by DnaA. However, in archaea, synchronous initiation of replication at multiple sites on the chromosome as well as recognition proteins homologous to eukaryotic ORC proteins suggests that there are similarities between archaebacterial and eukaryotic DNA replication to be explored.

Once the DNA is replicated and moved to opposite sides of the cell, the midcell septum forms to split the cell. At least 9 gene products are involved in this process including FtsZ, the prokaryotic tubulin homologue that forms a circumferential ring, FtsI, a peptidoglycan synthetase involved in septum formation, FtsL, whose function is unclear but is involved in ingrowth of the cell wall at the septum, and ZipA, which anchors the FtsZ ring. The ring contracts, pulling the membrane in with it. Eventually the membrane is pinched in enough to fuse and generate two completely separate cytoplasmic compartments. Other septation enzymes make cell wall components that fill in as the septum forms simultaneously with membrane/FtsZ contraction, and the cells separate.

The Eukaryotic Cell Cycle

Most eukaryotic cells undergo a reproductive cycle to generate either another copy of themselves or to generate gametes (sex cells), and in doing so require a complex mechanism to govern the safe and accurate replication of their much larger (than prokaryote) genomes. Immediately following mitosis, the newly created cells are in the G_1 phase. This is largely a growth phase, during which there is a lot of biosynthesis of proteins, lipids, and carbohydrates. However, there is no synthesis of new DNA at this time. G_1 is the longest of the cell cycle phases in many cell types, and most of the physiological activity of a cell happens during G_1. Following G_1, the next phase of the cell cycle is the S phase, during which synthesis of new DNA occurs. In other words, the genome is being replicated during this phase; thus at the end of S phase, the cell has twice the normal amount of DNA. After S phase, the cell proceeds into G_2, which provides an opportunity for the cell to perform a self-assessment and make final preparations (such as more cell growth, repairs of DNA) as necessary before it finally heads into mitosis. Mitosis, or M phase, is primarily (1) the breakdown of the nucleus, (2) re-distribution of the DNA to opposite sides of the cell, and (3) formation of two new nuclei around that DNA, and cytokinesis, the final splitting of the cell itself.

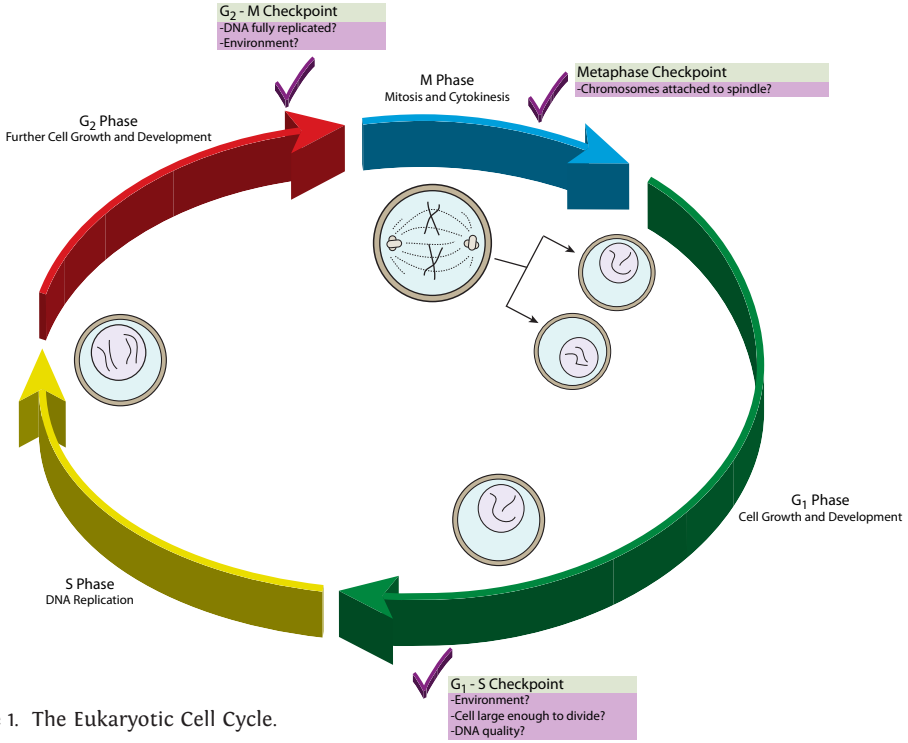


Figure 1. The Eukaryotic Cell Cycle.

As the cell progresses through the various phases of mitosis, and for that matter, the phases of the cell cycle overall, it does so in a specific and controlled manner, with checkpoints that "ask" if the cell is ready for the next step: is it big enough, is the DNA healthy, etc. so that the cell has the best chance of generating healthy daughter cells. For example, if the cell cycle runs too rapidly through each phase, then there is not enough time for the cell to build up its mass in preparation for reproduction, and that leads to abnormally small daughter cells, and potentially even daughter cells that are too small to survive. If a cell undergoes mitosis with damaged or mutated DNA, then that may increase the likelihood of a pathological mutation surviving and harming the organism by turning into a cancerous tumor.

Controlling the Cell Cycle

There are three major checkpoints for cell cycle control (fig. 1). The first regulates the transition from G_1 to S phase. Recall that G_1 can be a very long phase, even (in the case of G_0) as long as the lifespan of the cell. However, once the cell reaches S phase, it is committed to going through S, G_2, and M phases to reproduce. This is because once S phase has begun, there is more than the normal diploid complement of DNA inside the cell. Over time this would confuse the cell (e.g., by overexpression of duplicated genes) as it tried to use the DNA to direct RNA and protein synthesis, and it could become sick and die. The second major checkpoint regulates entry into mitosis. Once mitosis begins, most of the metabolic activity of the cell is shut down, and the cell concentrates its resources on dividing the nuclear and cellular material equally to support the life of both resulting daughter cells. If the cell needs more time to make final repairs on the DNA or even to bulk up a little, this checkpoint can hold the cell in G_2 a little longer for those things to happen. Finally, the third major checkpoint occurs during mitosis, and regulates the transition from metaphase into anaphase. Since the sister chromatids are being split apart and moved to opposite poles to form the new nuclei, it is important that all of them are perfectly lined up at metaphase and the proteins holding them together have dropped off. If they do not split evenly, the daughter cells will have abnormal numbers of chromosomes (aneuploidy) usually leading to deleterious consequences.

What is the molecular mechanism that regulates the progress of the cell cycle? While many of the checkpoint sensing mechanisms are still unclear, they seem to converge on two sets of proteins that act together to trigger cell cycle advancement. These proteins are known as the cyclins and the cyclin-dependent kinases (cdk). As the names suggest, the cyclins are proteins that regulate progression through the cell cycle, and

must be present in sufficient concentration to help activate the appropriate cdk. The cyclin-dependent kinase is the active, enzymatic, half of the partnership, and activates other enzymes by phosphorylation. Although the cyclins appear to be necessary for cdk activation, they are not sufficient, as there are intermediate phosphorylation and dephosphorylation steps required to activate the cdk after cyclin binding. Both the cyclins and the cdk's are families of related proteins, and they can combine in different ways to govern particular points in the cell cycle (fig. 2). Interestingly, the intracellular level of cdks is fairly constant. The level of cyclins, on the other hand, fluctuates dramatically depending on the state of the cell with respect to the cell cycle.

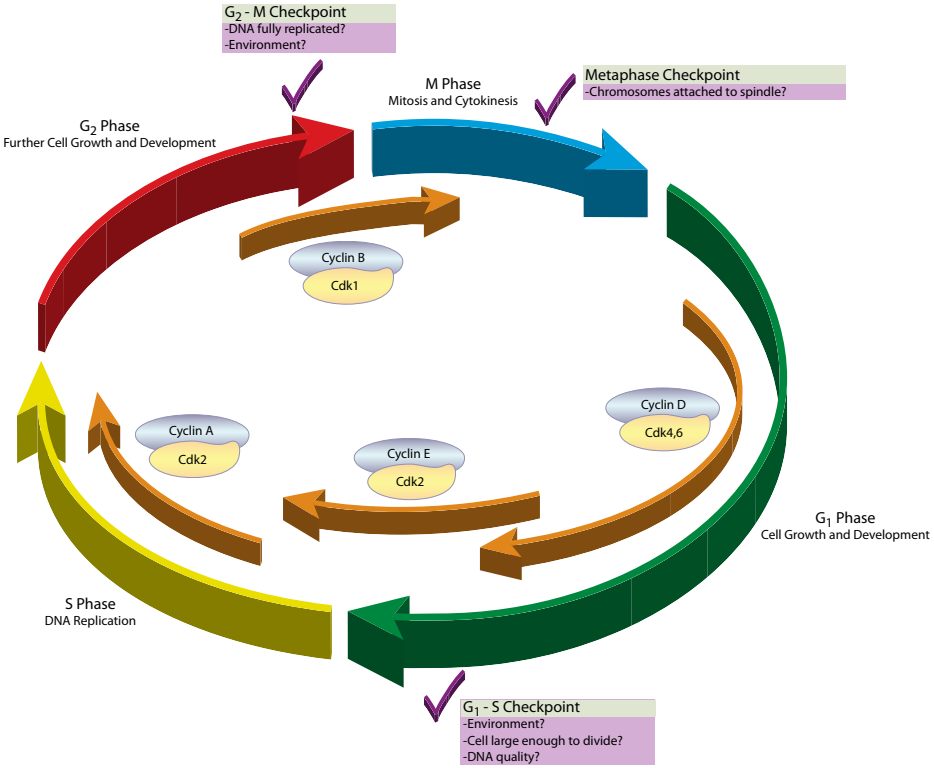


Figure 2. Cyclins are involved in control of the cell cycle.

The methodology of some of the early experiments is perfectly suited to explaining how this works. The seminal paper in this field was a 1971 paper in J. Exp. Zool. by Masui and Markert. In it, they examined frog (Xenopus laevis) eggs that were arrested at G_2. The oocytes arrest for about 8 months naturally in order to build up the mass needed to start a new organism once it has been fertilized. The basic question being asked is what is causing the eggs to come out of G_2 and into M phase? It was already known

that the hormone progesterone can trigger this transition, but what are the intracellular players in the change in cell state? Masui and Markert decided to test whether there was a cytoplasmic molecule that was responsible. They took a small amount of cytoplasm from an M-phase egg and injected it into a G_2-arrested egg. This triggered the maturation of the G_2-arrested egg and pushed it into M phase, even without progesterone. The activity was called maturation promoting factor (MPF), and was hypothesized to be a soluble, cytosolic protein.

In later experiments, other investigators attempted to find the specific protein trigger, and from there, presumably, the rest of the mechanism. Fractionating the M-phase oocyte cytoplasm by column chromatography, a protein, named cyclin B, was found to rise and fall in concentration in direct synchronization with MPF activity. Furthermore, addition of cyclin B alone was sufficient to rescue MPF activity from M-phase cytoplasmic extract that had been depleted by RNase treatment (preventing synthesis of any new proteins, including cyclin B, and abolishing MPF activity). This clearly places cyclin B in the forefront of the maturation mechanism, but there was one major issue: cyclin B had no enzymatic activity. How was it effecting the changes needed for progress from G_2 to M phase?

This problem was answered by experiments on a very different organism, the fission yeast, *Schizosaccharomyces pombe*. Because they have a very short cycle time, a relatively small genome, and they can be given random mutations *en masse* by irradiation or chemical treatment, yeast are excellent model organisms for many types of biological study. After random mutation of a population of yeast, they can be screened for mutations of particular types, such as cell division cycle (cdc). When the mutations are sequenced and identified, they are often named by the type of mutation and order of discovery. Cdc2, it turns out, showed two interesting phenotypes when mutated in opposite directions. Mutations that knocked out function of cdc2 caused the formation of extremely large yeast that do not undergo cell division, while mutations that made cdc2 overactive caused the formation of rapidly dividing very small cells. The interpretation was that when cdc2 is missing or inactive, the cells cannot progress to mitosis, so they stay in G_2 accumulating bulk material in preparation for a cell split that never comes. Conversely, when cdc2 is overactive, it drives the cell quickly into mitosis, even if it has not been in G_2 long enough to synthesize enough mass to form two normal-sized cells. This ties cdc2 nicely to cell cycle regulation, and it even has an enzymatic activity: it is a kinase. This made it a perfect candidate as a first-order coordinator of cellular events because phosphorylation is fast, phosphorylation usually activates some other enzyme, and kinases usually act on an array of targets, not just one. So we now have a cyclin (identified as cdc13 in S. pombe) and a cyclin-dependent kinase that work together to promote cell cycle progression into M phase.

Activation and inactivation of the cyclin-cdk complex

As more mutant yeast were being screened for changes to their cell cycle, two other genes were found in which mutations gave rise to similar phenotypes. Nonfunctional cdc25 or overactive wee1 mutants generated the overly large cells with a single nucleus, and conversely, overactive cdc25 or inactive wee1 generated many severely undersized cells. Both cdc25 and wee1 gene products interact with cdk, and in fact, they are positive and negative regulators of cdk, respectively. Acting together with one more enzyme, CAK (cdk-activating kinase), they activate the cdk (fig. 3). Using the mitotic cyclin/cdk complex as an example, the cyclin (cdc13) and cdk (cdc2) come together to form an inactive complex. The cdk is then phosphorylated by wee1, a kinase. The phosphate it puts on tyrosine-15 is needed for the rest of the activation sequence, but it is inhibitory: it actually *prevents* final activation. But once Tyr-15 is phosphorylated, CAK can phosphorylate a neighboring threonine (Thr-161), which is required for activation. Finally, cdc25, a protein phosphatase, removes the phosphate on Tyr-15, allowing activation of the cdk by the phosphorylated Thr-161, and the MPF is finally on its way. There is self-amplification of the activation as well, because one of the targets of MPF is cdc25, so there is a positive feedback loop in which the activity of cdc25 is upregulated by phosphorylation.

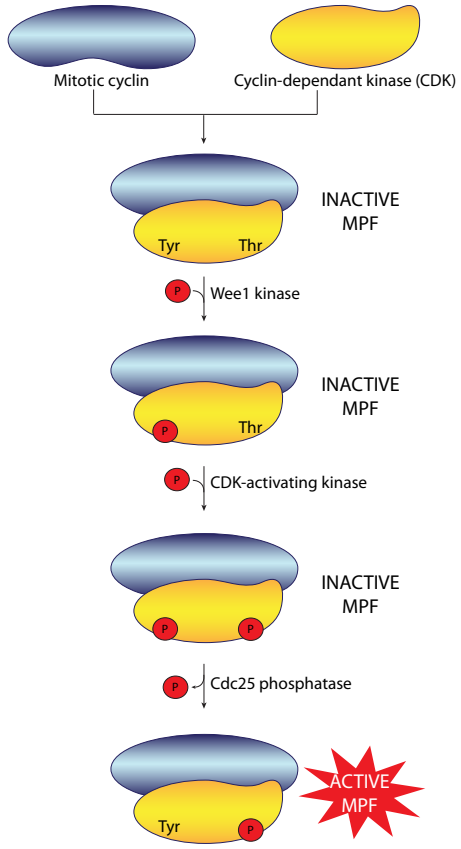


Figure 3. Activation of mitotic cyclin/cdk complex.

As you will see in a later section of this chapter, MPF performs many functions, some of which prevent progress of mitosis past anaphase. Therefore, there must be a way to turn off MPF (and for that matter, any cyclin/cdk complex) quickly and completely when the cell reaches the appropriate stage of the cell cycle. This is borne out by time-course studies of MPF activity, which show a precipitous drop in activity in anaphase. This coincides with a depletion of the cyclin B (cdc13 in S. pombe) due to a combina-

tion of turning off transcription of the gene, and specific proteolytic degradation. The degradation pathway is now well understood, and is an interesting example of a sort of feedback regulation.

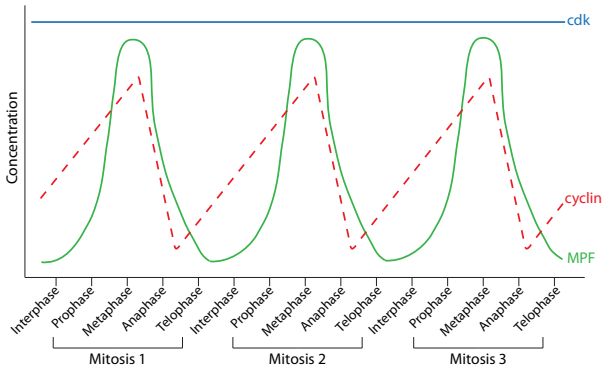


Figure 4. MPF activity and cyclin B protein expression rise as the cell enters mitosis but drop just before anaphase. However, cdk levels remain steady throughout the cell cycle.

Essentially, MPF ensures its own destruction: one of its phosphorylation targets is cdc20. Upon phosphorylation, cdc20 is activated and then activates anaphase promoting complex (APC). APC is a ubiquitin ligase (type E3) that polyubiquitinates the cyclin of the MPF complex, making it a target for proteolytic degradation by a proteosome. Note that only the cyclin is destroyed, while the kinase is left alone. Without the cyclin, the kinase is inactive and must wait for cyclin levels to rise again before it can be reactivated by a fresh round of phosphorylation and dephosphorylation.

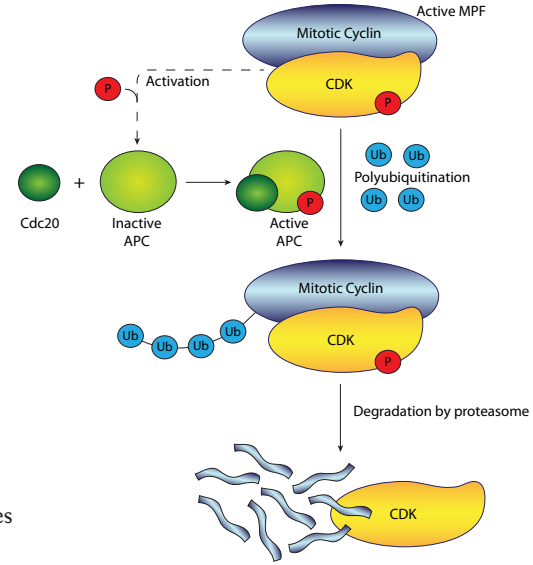


Figure 5. MPF and other cyclin/cdk complexes are inactivated by destroying the cyclin.

G_1/G_0 phase

The G_1 phase is the state a cell is in immediately following cytokinesis. At that point, the cells will be somewhat undersized, and need to take up materials and energy sources, and convert them to cellular components in order to support the eventual cell division. During this time, the cell goes about doing its "normal" business - an endocrine cell makes and secretes hormones, an intestinal epithelial cell absorbs nutrients from the gut and passes them on to the bloodstream, a neuron conducts signals, etc. Most types of cells spend the majority of their cycle in G_1, although there are exceptions, such as the frog oocytes mentioned earlier. The length of G_1 is generally constant for a given cell type under normal conditions, but can vary greatly between different cell types. Post-mitotic cells, which have left the cell cycle and will no longer divide, are in G_1 until they die, barring reactivation of the cell cycle by stress conditions. This continuous G_1-like state is referred to as G_0.

For those cells preparing to move from G_1 into S, cyclins D and E, and cdk 2, 4, and 6 predominate, with activation of cyclin D complexes preceding activation of cyclin E complexes. Two major questions are asked by the cell: is the DNA undamaged and complete, and is the extracellular environment favorable for cell division? The cellular sensors for these conditions then link to cyclin complexes effect restriction points on cell cycle progression. The extracellular environment questions can be a tricky one, because this can include more than just assessment of nutrient availability or predatory threats; it can also be a requirement for an external trigger such as a mitogenic hormone or paracrine signal. In fact, nearly all normal animal cells require an extracellular signal to progress through the G_1/S checkpoint. The cyclin E/ cdk2 combination is the principal regulator of entry into S phase and DNA replication.

The active cyclin E/cdk2 complex phosphorylates the tumor suppressor protein Rb (retinoblastoma), which causes E2F to translocate to the nucleus and turn on genes needed for entry into S phase.

S phase

The mechanisms of DNA replication were discussed in chapter 7. It is important to note that once a cell has entered S phase, it has essentially committed to going through cell division. Cells do not cope well with extra copies of chromosomes, and a cell that went through S phase without going through mitosis would likely have major malfunctions in gene regulation. For similar reasons, the cell must only undergo DNA replication once per cell division. The cyclinA/ cdk2 complex plays a key role in initiation of replication by activating the pre-replicative complex. It also phosphorylates cdc6, causing it to dissociate from the ORC, and consequently the rest of the pre-RC. This prevents immediate re-use of this origin of replication, and since the phosphorylation of cdc6 allows it to be recognized by a ubiquitin ligase complex, it is tagged for proteolysis.

In addition to DNA replication, S phase is also the cell cycle stage in which centrosomes are duplicated in animal cells. The cyclin E/cdk2 combination licenses the duplication of centrosome, phosphorylating nucleophosmin, which then dissociates from the centrosome. This helps to trigger the centrosome duplication. Nucleophosmin does not reassociate with centrosomes until telophase, when it is no longer phosphorylated. Plk4 (Polo-family kinase 4) activity is necessary for centriole duplication, and appears to initiate the centriole assembly mechanism.

G_2 phase

The G_2 phase begins when DNA replication has completed. Having said that, before the cell is allowed out of G_2 and on to M phase, it must pass a DNA fidelity checkpoint, ensuring that not only has replication been fully completed, but that there are no major errors. G_2 is a relatively short phase (compared to G_1) in most cell types, and it is spent building up energy and material stores for cell division and checking the DNA. If everything is ok, and the cyclin B/ cdk1 complex has been activated, the cell proceeds to M phase.

Mitosis

Mitosis consists of prophase, metaphase, anaphase, and telophase, with distinct cellular activities characterizing each phase. This completes the duplication of the nucleus, and is followed by cytokinesis, in which the cell divides to produce two daughter cells.

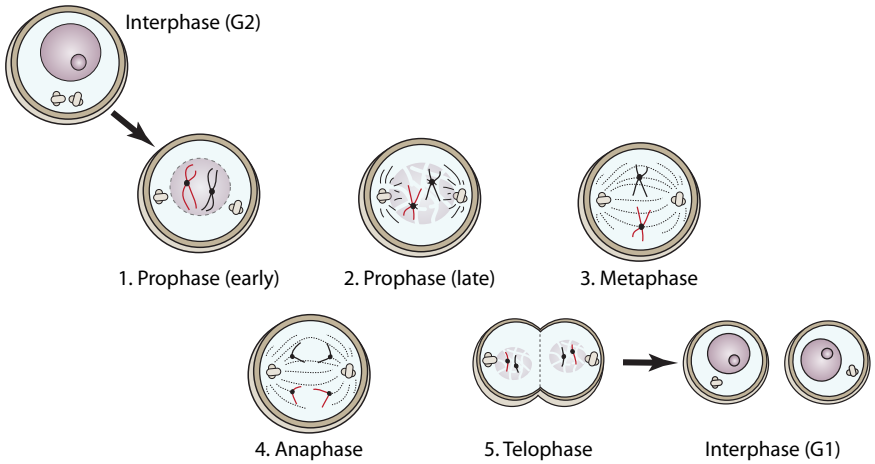


Figure 6. Mitosis. During mitosis, the nuclear envelope breaks apart to allow the spindle access to the chromosomes. Once they have been moved to opposite ends, the nuclear membrane reforms around each set. Finally, cytokinesis divides the cell into two new daughter cells.

The ubiquitin ligase complex, SCF, is made up of three major proteins and several minor species. Skp1 (S-phase kinase-associated protein 1) can be an RNA polymerase elongation factor, but in this complex links the other two proteins together. Cul1 (Cullin 1) is an E3 type ubiquitin ligase. Finally, an F-box family protein like Rbx1 (Ring-box 1), that heterodimerizes with cullin-1 and may also recruit E2 ubiquitinating enzyme.. In addition to cdc6, it also recognizes and ubiquitinates CKIs (cyclin complex kinase inhibitors) such as p27, which is involved in processes such as DNA repair and error-checking.

Prophase is the preparation of each component for this complex cellular dance. The DNA condenses (it is wrapped around itself tightly to make it a smaller and stronger package) so that it is less susceptible to breakage during movement across the cell. In doing so, most of the DNA becomes transcriptionally inactive. The Golgi bodies and the endoplasmic reticulum begin to break apart into membranous vesicles that can be more easily and evenly distributed across the cell so that both daughter cells receive about the same. The centrosomes (in animal cells) move from their original position near the nucleus toward opposite sides of the cell, to establish the poles of the mitotic spindle.

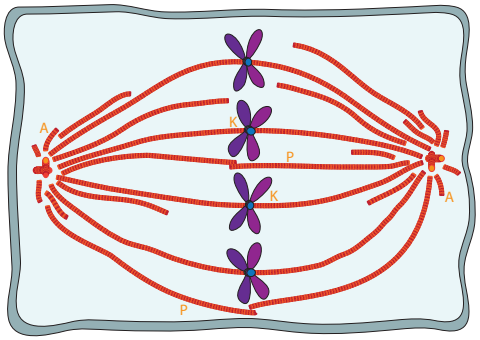


Figure 7. The mitotic spindle. The spindle is made of microtubules that originate from the centrosomes, which have migrated to opposite sides of the cell. There are three types of spindle microtubules: the kinetochore microtubules (K), polar microtubules (P), and astral microtubules (A).

MPF phosphorylates microtubule motor proteins and microtubule associated proteins (MAPs) to alter the normal microtubule dynamics and allow the massive reorganization into a mitotic spindle to occur. For example, one target of MPF is PRC1, a bundling protein that is inactivated by phosphorylation, thus allowing individual microtubules to move to new locations more easily than a large bundle could. Other effects are inactivation of stabilizing MAPs, which leads to greater lability of microtubules due to increased incidences of catastrophe. The motor protein targets of MPF are in the kinesin family and the phosphorylation is necessary for some of them to bind to the mitotic spindle.

Prometaphase is sometimes considered a separate phase but is also referred to as late prophase, and is primarily defined by the breakup of the nuclear envelope. This process is induced by MPF phosphorylation of the nuclear lamins. Adorned with negative charges from the phosphates, the lamins refuse to associate with one another any longer, leading to the breakdown of the nuclear lamina. As the lamins dissociate, the nuclear envelope remains bound to them, and fragments. This nuclear fragmentation must happen so that the mitotic spindle can reach inside and attach to the chromosomes.

Some of the microtubules of the mitotic spindle attach to the chromosomes via the kinetochore proteins, which link the spindle microtubules to the centromere region of each chromosome. These are known as kinetochore microtubules (fig. 8). There are two other types of microtubules in the mitotic spindle (fig. 7): the polar microtubules that reach across the cell and interact with one another to help maintain the separation of the centrosomes and defining the overall length of the spindle, and the aster microtubules that are generally short, radiating out from, and stabilizing the centrosome. Remember that the DNA replicated earlier in S phase, and thus sister chromatids are still partially attached. Visually, the centromere region appears narrower or more compressed than the rest of the chromosome, and generally lies near the middle. The centromere contains repeated sequences that are involved in kinetochore binding and assembly.

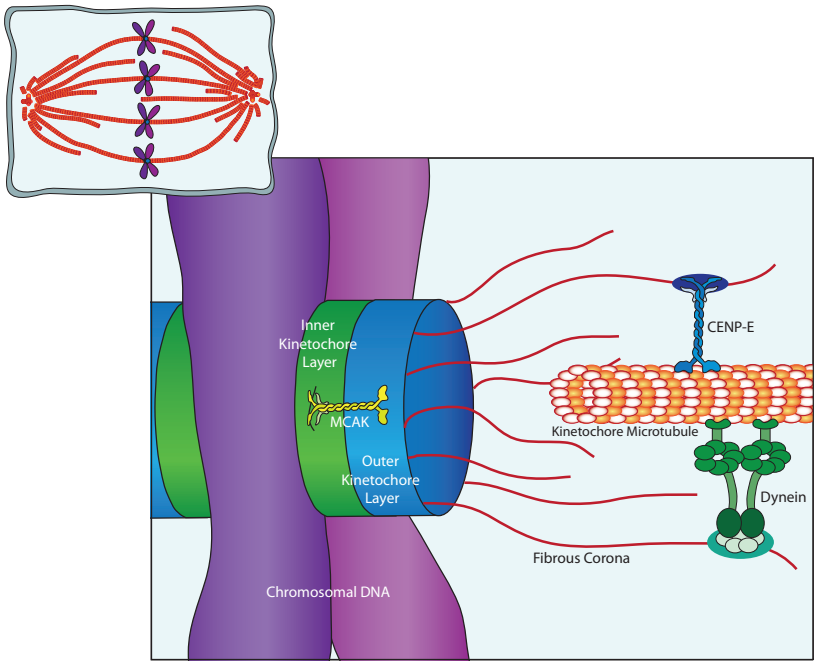


Figure 8. The kinetochore assembles on the centromere of the chromosome. Spindle microtubules attach to the fibrous corona of the kinetochore through kinesins and dyneins.

The kinetochores attaching to the centromere DNA are trilaminar protein structures consisting of an inner layer, an outer layer, and a fibrous corona. The kinetochore microtubules of the mitotic spindle are primarily attached to the fibrous corona. As depicted in the figure, it is attached through CENP-E, a kinesin, and dynein motor proteins that bind along the barrel of the microtubule. In fact, sometimes the first contact between a chromosome (via the kinetochore) and a spindle microtubule is somewhere

In primates, the repeating motif is known as alpha satellite DNA, which is made of multiple instances of tandem repeats of a core ~170bp sequence over a centromeric DNA span over a megabase in length. Similar repeats are found in various other vertebrates as well. In other eukaryotes, the size and sequence may vary; for example, much shorter repeats of ~5bp are found in centromeric DNA measuring 200-600kb in Drosophila chromosomes, and S. pombe has centromeric DNA well under 10kb.

in the middle of the microtubule, and a combination of microtubule dynamics and motor protein activity move the chromosome to the distal end of the microtubule. This is facilitated by MCAK (mitotic centromere-associated kinesin), which is associated with the kinetochore core proteins and plays a role in depolymerizing microtubules near the (+) end.

As the nuclear envelope is breaking apart, the mitotic spindle microtubules are undergoing increased dynamic instability, cycling between periods of growth spurts (polymerization) and rapid shortening (catastrophic disassembly), searching for chromosomes to connect to. Once the kinetochore microtubules connect to the chromosomes, the microtubule dynamics shift. The microtubule will primarily undergo shortening if it is beyond the center of the spindle and primarily lengthening if it is short of center. Since eventually each set of sister chromatids is connected to microtubules on both kinetochores, each chromatid is connected to one shortening and one lengthening microtubule. As the chromosomes approach the center of the mitotic spindle, the rate of microtubule shortening/lengthening slows. The sister chromatids are pushed and pulled by the spindle microtubules until they are all lined up along the midline of the mitotic spindle, which in most (but not all) cases is also the midline of the cell. Once they are all lined up, the cell is considered to have reached metaphase. Unlike the other phases, metaphase is a relatively static phase - it is a checkpoint for lining up the chromosomes.

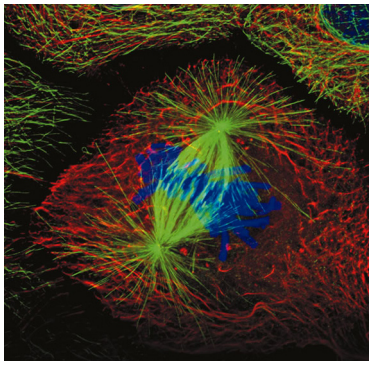


Figure 10. A cell at metaphase. Microtubules are stained green, f-actin is stained red, and chromosomes, with centromeres lined up along the midline, are stained blue. Note the surrounding cells, which are not in mitosis, with their MT and MF cytoskeletons more overlapped. This photo released to public domain by the US goverment.

The chromosomes must be properly aligned to ensure that both daughter cells receive the proper complement of chromosomes. How does the cell know when the chromosomes have reached the center of the spindle? An elegantly simple experiment demonstrated that the general mechanism is a tension check - if the two microtubules connecting to the pair of sister chromatids from each side are of the same length, they should be exerting equal tension on the chromosomes. If the microtubule-kinetochore connection is severed at metaphase, the cell will be prevented from progressing (Nicklas, R.B., et al, *J. Cell Biol.* **130**: 929-39, 1995). However, if an equivalent tension is applied by tugging on the chromosome with a glass microneedle, progression of mitosis is restored!

The transition from the interphase microtubule cytoskeleton to a mitotic spindle require a number of molecular motors to move the centrosomes, align the microtubules, and expand the spindle. These are depicted in fig. 9. Initially, as the duplicated centrosomes move away from each other along with some of the cytoskeletal microtubules, the microtubules will interact at various angles. Because the polar microtubules that help to expand or maintain the spindle width must ineract in parallel, cytoplasmic dyneins bind to the eventual polar microtubules and by moving one along the other, bring them into parallel (9a). Once in that position, BimC and other kinesins take over as the primary motors along polar microtubules. They create an outward pushing force by holding onto a microtubule facing one direction, and driving along a parallel MT facing the opposite direction towards the (+) end (9b). Finally, cytosolic dyneins attached to cortical cytoskeleton pull on the astral microtubules, which pulls the spindle ends further from center (9c).

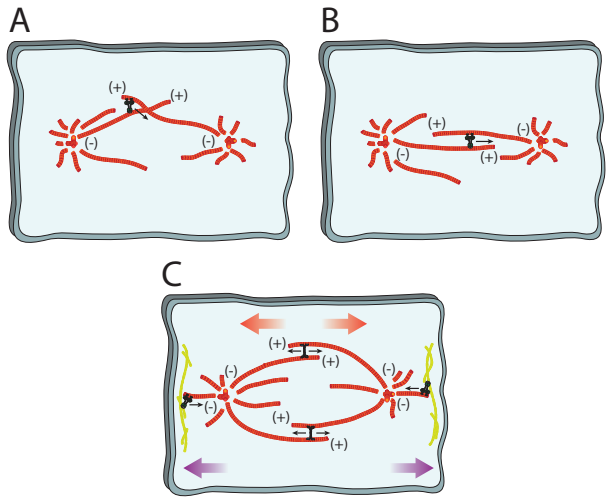


Figure 9. Molecular motors set up the mitotic spindle.

In fact, there appear to be two mechanisms at work: the bub1/bub2 system works in the tension sensing pathway, while another metaphase protein, mad2 appears to be important in suspending mitosis upon disconnection of the kinetochore with the spindle microtubule.

In addition to the tension check, there is another condition that must be met for continuation of mitosis: the MPF must be inactivated. As outlined earlier, MPF in part leads to its own inactivation by activating the anaphase-promoting complex (APC), which polyubiquitinates the cyclin, leading to its destruction and thus MPF-cdk inactivation. APC also tags securin for destruction. Securin is a protein that binds and inhibits the proteolytic enzyme, separase, the activation of which is needed to allow the sister chromatids to separate, which in turn, is necessary for anaphase to proceed.

Barring pathological situations, if and only if the chromosomes all line up at the metaphase plate will the cell proceed to the next stage of mitosis: anaphase. The sister chromatids separate and are pulled toward opposite poles of the mitotic spindle. Somewhat perversely, even as the chromosomes move towards the spindle poles, the poles themselves move outward slightly. Separation of the sister chromatids requires the dissociation of the molecular "glue" holding them together: the cohesin proteins. The cohesins bind to both molecules of DNA and hold them together shortly after replication back in S phase. As anaphase approaches, the enzyme separase is activated, which then cuts the cohesin molecules. Once all of the cohesin molecules are cut, the sister chromatids can finally be separated. The removal of the cohesins proceeds roughly inwards from the distal points of the chromosomes to the centromere, which is generally the last region of attachment.

A cohesin is a multimer of four subunits, Scc1, Scc3, Smc1, and Smc3 in yeast. An additional protein has also been observed in Xenopus. The SCC1 protein is cleaved by separin in yeast, but in metazoans, SCC1 may be removed from chromosomes by another method as well. It is phosphorylated, which decreases its affinity for DNA, and may expose a site for separase-catalyzed hydrolysis.

Anaphase can actually be divided into two stages, sometimes referred to as early and late or A and B. At first, the kinetochore microtubules are shortening from both ends, and kinesin-family motors pull the microtubules back toward the spindle poles. As late anaphase starts, polar microtubules elongate, and an additional chromatid-separating force is applied by kinesin-family motor proteins [kinesin-5] that push the polar microtubules against one another to increase the separation between the poles. Dynein-family motors help to direct movement of the poles as well, through their attachment to the aster microtubules and the cortical (peripheral) cytoskeleton.

Separase also promotes anaphase by activating Cdc14, a phosphatase needed to dephosphorylate the cdk substrates that had been phosphorylated by the cyclin-cdk complexes of early mitosis. In addition, Cdc14 is also required for cytokinesis in the yeast S. cerevisiae and nematode C. elegans.

When both sets of chromosomes arrive at their respective poles, telophase begins. Technically, it was slowly building up since anaphase: when MPF was inactivated by APC, its ability to phosphorylate nuclear lamins was ended. Protein phosphatases in the cell remove the phosphate groups, allowing the lamins to once again interact with one another, and by telophase they are reconstituting the nuclear lamina and the nuclear envelope. Since the lamins and other nuclear membrane proteins also interact with DNA, the nuclear membrane fragments dispersed back in late prophase now coalesce around each set of DNA to form the new nuclear envelopes. The other fragmented membranous organelles (ER, golgi) also start to re-form. By the end of telophase, the product is a single large cell with two complete nuclei on opposite sides. The next and

last step, cytokinesis, splits the cell into two separate and independent daughter cells. In animal cells, cytokinesis is similar to the tightening of a drawstring in the middle of the cell, pulling the "waist" in until all edges meet, and two separate cells result. This contractile ring is composed of actin (structural) and myosin (motive) subunits. These proteins, using ATP for energy, ratchet themselves closer and closer together similar to the actin-myosin "power stroke" described for muscle cell sarcomeres, also primarily made from actin and myosin. This mechanism is universal for animal cells, but the placement of the ring is not always in the center of the cell. The ring often coincides with the center of the cell, but is in fact positioned by the metaphase plate (i.e. the center of mitotic spindle). The most obvious example of a metaphase plate that does not coincide with the center of the cell is found in the formation of egg cells. Because the purpose of an egg cell is to provide all of the material necessary to make a viable new organism upon fertilization (the sperm contributes negligible biomass beyond the genetic material), it divides asymmetrically, with the mitotic spindle located far to one side of the cell (fig. 19). When cytokinesis occurs, one daughter cell, the presumed oocyte, is very large, while the other cell, called a polar body, has minimal cytoplasmic material surrounding the nucleus. The contractile ring works in animal cells because the cell membrane is flexible. In plant cells, the cell membrane is firmly attached to a rigid cell wall, and thus cannot be pulled in. So, the plant cell ingeniously builds a wall down the middle of the cell using specialized vesicles that originate from part of the Golgi, and which contain the materials necessary to form a cell wall. The vesicles travel along the *phragmoplast*, a structure built from the mitotic spindle microtubules, and as the vesicles line up along the middle of the cell, they begin to fuse to form bigger vesicles and then a large disk-like vesicle, the cell plate. Eventually they reach the cell membrane itself, and fusing with that leads to formation of a new cell wall, and two complete and independent cells.

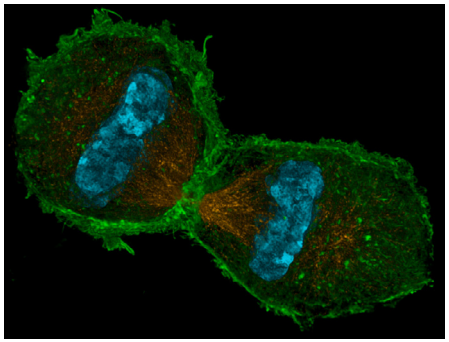


Figure 11. Telophase/ Cytokinesis. The contractile ring and other actin structures are stained green, the microtubules are orange, and the chromosomes are blue. Photo released to public domain by US government.

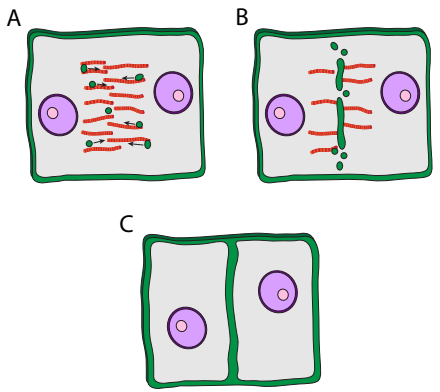


Figure 12. Cytokinesis in plant cells. Golgi-derived vesicles filled with cell wall material travel along the phragmoplast and fuse in the center to form a new cell wall.

The contents of the vesicles traveling along the phragmoplast are not well described. Callose, a glucose polysaccharide with β1-3 linkages is known to be present in the developing cell plate, but has not been found in the Golgi or vesicles. Interestingly, once the cell plate has fused completely with the existing cell walls, callose gradually disappears. It is thought that the same enzyme system that synthesized callose may switch to synthesizing cellulose as the cell plate matures.

Cell Death

A cell may die either intentionally (usually referred to as apoptosis or programmed cell death, though also once known also as "cellular suicide"), or unintentionally (necrosis). The microscopic observation of these two processes shows strikingly different mechanisms at work. In apoptosis, the cell begins to shrink and lose shape as the cytoskeleton is degraded, then the organelles appear to pack together, except for the nucleus. Inside the nucleus, the chromatin condenses and attaches to the nuclear envelope, which then loses its integrity and starts to break apart. The cell membrane begins to show irregularities, descriptively known as blebs, and eventually, the cell breaks apart into vesicles that are neatly cleaned up by phagocytes drawn to the site by apoptotic signals emitted by the dying cell. Necrosis, on the other hand, is quite literally a mess. The cell appears to swell and the plasma membrane begins to lose its integrity. It is soon catastrophically leaking cytoplasm, and leaves behind cell debris that can accumulate and trigger necrotic death of adjacent cells.

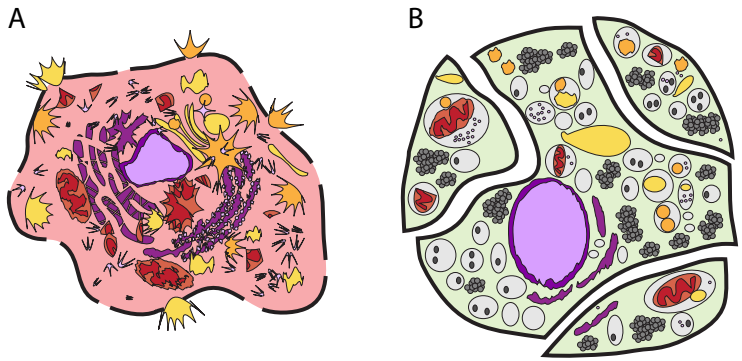


Figure 13. (A) A cell underdying by necrosis is disorganized, generally bursts and leaks its contents. (B) A cell undergoing apoptosis first subdivides itself, digesting itself in an orderly fashion and compartmentalizing everything for scavenging by phagocytes.

Apoptosis is ultimately put into action by a cascade of caspases, a family of proteolytic enzymes. This family of enzymes is generally produced as proenzymes that are activated by other members of the caspase family. Thus a cascade effect occurs, after the initial trigger activating one set of caspases, they can then cleave a variety of proteins including procaspases that are thereby activated and can hydrolyze even more proteins, including yet another type of procaspase, and so on. Of course, other enzymes are also activated and participate by widening the response, activating other groups of proteases and apoptotic enzymes. Triggering the apoptotic cascade is usually one of two general pathways: an internal trigger, arising from damage to the mitochondria, and an external trigger, started by binding an extracellular signal molecule to activate

a “death receptor”. Although there are many variations on both triggers, they follow similar paths to the examples we will use here.

If you recall the section on electron transport in oxidative phosphorylation, then you may also recall the soluble electron carrier, cytochrome c. This protein is exclusively found in the mitochondrial matrix under normal circumstances, so its presence in the cytoplasm can be taken to indicate mitochondria in distress. Given the importance of mitochondria in providing the energy for most aerobic cells to carry out their normal life, such distress is an early indicator that the cell will die soon. The diagram at right shows a sample pathway that can cause cytochrome c leakage from the mitochondria, but mitochondria can also just “get old”, and if the cell is “programmed” (by transcription factors) not to replace failing components, then as the mitochondrial membranes lose integrity and allow cytochrome c out, it is a clear signal to initiate termination protocols, to use the parlance of science fiction novels. The cytochrome c is bound by APAF-1 (apoptotic protease activating factor 1) which oligomerizes to form an apoptosome made of 7 APAF-1 moecules and 7 cytochrome c molecules. The apoptosome binds and activates procaspase-9 to initiate a caspase cascade that continues with activation of procaspase-3. When the mitochondria leaks cytochrome c, it also leaks another apoptotic protein, SMAC/Diablo. This protein, among other functions, inhibits IAP (inhibitor of apoptosis) -family proteins. The IAP proteins normally inhibit caspase activation both directly and indirectly to prevent cell death, and SMAC/Diablo blocks that inhibition.

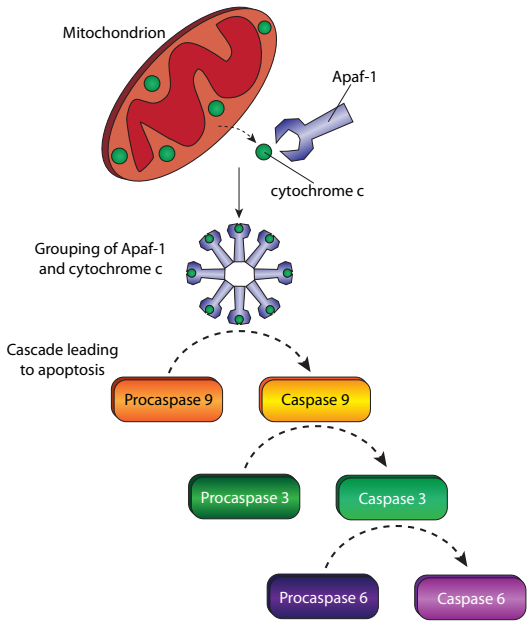


Figure 14. Apoptotic signaling cascades may be initiated by leakage of cytochrome c into cytoplasm.

When death receptors are activated, the subsequent caspase cascade does not involve the mitochondria or APAF-1. The best studied case, FasR (Fas receptor) activates caspases 2, 8, and 10 by clipping procaspases and by releasing caspases from inhibiting complexes. These activate caspases 3, 6, and 7, which leads to the final stages of apoptosis. In both internally and externally triggered apoptosis, the final steps are the same: some of the final targets of the caspases are the nuclear lamins and ICAD (inhibitor of cas-

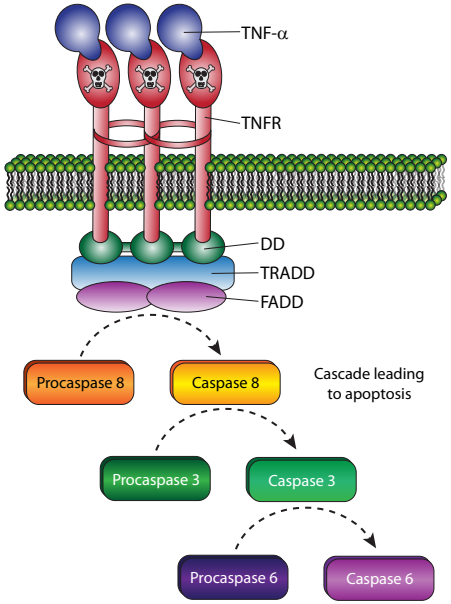


Figure 15. Apoptotic signaling cascades may be started by external activation of a "death receptor" such as FasR.

pase-activated DNase). Destroying the nuclear lamins leads to fragmentation of the nuclear envelope, while removing ICAD activated the caspase-activated DNase (CAD) which then begins to digest the DNA.

Why does the apoptosis mechanism exist? There are two major (and many other) reasons for apoptosis. The first is developmental. In the development of an organism, the most effective strategy is often to have overgrowth of cells that are then pruned back to the proper formations. Examples of this are the apoptotic death of tissue between initially connected fingers and toes (we humans start with webbed fingers and toes embryonically), and death of unconnected or improperly connected neurons. The latter case also illustrates a fundamental principle in mammalian cell biology, and most other vertebrates as well: cells require signals (trophic factors) to stay alive. In this example, the neurons that do not make proper connections to a target cell do not receive the necessary trophic factor (secreted by the target). This leads to apoptotic death of the unconnected neuron. In fact, if apoptosis is blocked due to mutation to a gene in the pathway, there is severe overgrowth of the brain and spinal cord, causing serious malfunction and craniofacial deformities. Thus in development, apoptosis is necessary to control the growth of different parts of a metazoan organism.

The other major function for apoptosis is to kill dangerous cells. In some cases, these may be cells infected by a pathogen. In others, the cells have accumulated mutations that do have affected the DNA error-correction system or cell-cycle checkpoints. When the former occurs, each generation has an increased likelihood of even more mutations. It is important to activate apoptosis in such cells before they have a chance to acquire errors that removes all cell cycle checkpoints, allowing unchecked cell proliferation. This could lead to tumor formation and potentially cancer (see next chapter). When such cells need to be killed for the benefit of the organism, it may happen by the triggering of an internal sensor such as mitochondrial damage, or by external means, such as an immune system cell recognizing an infected cell.

Meiosis

In metazoa, there are two situations in which a cell gives rise to daughter cells. The first, and by far most common, is mitosis. The second is meiosis. Meiosis is the process by which gametes (sex cells) are generated. Animals and plants are generated by sexual reproduction (if this is news to you, please consider majoring in something other than biology). These organisms start life through the fusion of two cells: a sperm and an egg. Both contribute genetic material to the new organism. In order to maintain the proper number of chromosomes in each generation, the gametes each contribute one set of chromosomes, so that the fertilized egg and all other cells in the organism have two sets of chromosomes — one from each parent. The purpose of meiosis, and its primary difference with mitosis, is not generating daughter cells that are exact replicates, but generating daughter cells that only have half the amount of genetic material as the original cell.

Let us take a look at this situation selfishly: meiosis in human beings. Almost every cell in your body has a nucleus containing 46 chromosomes, a set of 23 from your father, and a set of 23 from your mother. The only exceptions are the gametes: the spermatocytes in men and the oocytes in women. The somatic cells are said to be 2n or diploid, that is having 2 sets of chromosomes, and the gametes are 1n or haploid, having only one set of chromosomes. Sometimes, meiosis can be a little confusing to students because it occurs in the same part of the cell cycle as mitosis, which is to say after G_2. Because of this, the cell entering meiosis actually has 4 sets of chromosomes, since the DNA has already undergone replication in S phase.

Meiosis consists of two consecutive meiotic divisions each of which has phases similar to mitosis: prophase, metaphase, anaphase, telophase, and each of which finishes with complete cytokinesis. Note that immediately following meiotic telophase I, the cell divides, and both daughter cells are immediately in prophase II. There is no intervening G_1, S, or G_2 phase.

Prophase I of meiosis begins very similarly to prophase of mitosis: MPF (mitotic-cdk) activation, chromosome condensation, spindle formation and nuclear envelope breakdown. However, compared to mitosis, meiotic prophase I lasts for a very long time and can be subdivided into five stages: leptotene, zygotene, pachytene, diplotene, and diakinesis. During leptotene, the two sets (maternal and paternal) of sister chromatids for each chromosome condense, align and form a structure known as a bivalent. To clarify, this bivalent consists of four copies of a given chromosome: two copies each of the maternal chromosome and of the paternal chromosome. Because the maternal and

Mature red blood cells contain no nucleus, and some muscle cells, while multinucleated because they form from the fusion of several myoblasts, nevertheless have 46 chromosomes in each of the nuclei.

Polyploidy, while uncommon in humans, is a normal state for many organisms. The frog, *Xenopus laevis*, a common research animal, is tetraploid.

paternal versions of a given chromosome are kept in extremely close proximity for an extended period of time, there is a greater chance of a *recombination*, or crossing over and exchange of homologous pieces of each chromosome.

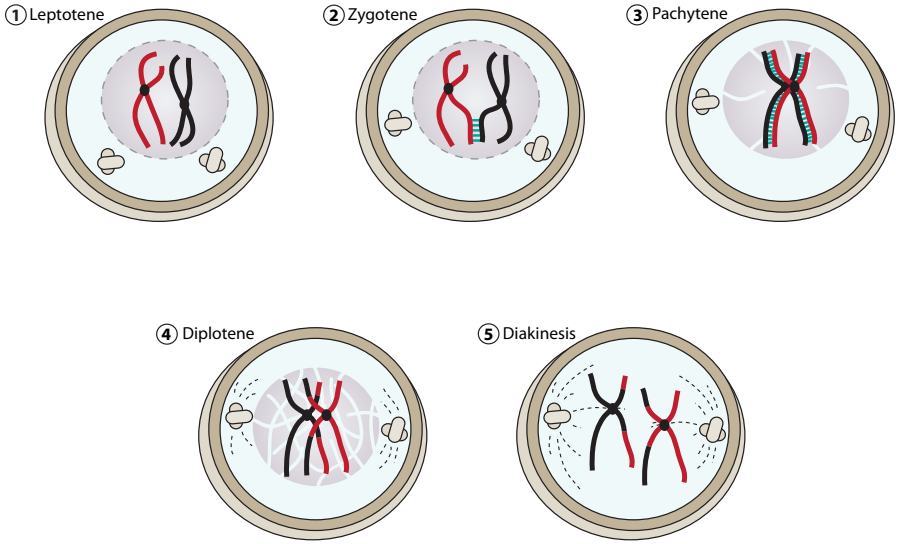


Figure 16. The five stages of Meiotic Prophase I.

Recombination occurs when a piece of the paternal chromosome is swapped for the homologous piece of DNA on the matching maternal chromosome (or vice versa). Note that sister chromatids (i.e. exact copies) do not recombine - only homologous non-sister chromatids can recombine. Obviously, this kind of a DNA swap must be done carefully and with equivalence, so that the resultant DNA on each side contains all the genetic information it is supposed to, and no more information than it is supposed to. In order to ensure this precision in recombination, the non-sister homologous chromatids are held together in a synaptonemal complex (SC). This ladder-like complex begins to form in the zygotene stage of prophase I and completes in pachytene. The complete SC consists of proteinaceous lateral elements (aka axial elements) that run along the length of the chromatids and a short central element composed of fibrous proteins forming the rungs of the ladder perpendicular to the two lateral elements. The central element is formed of transverse filament dimers that interact with one another in offset fashion, as well as with the lateral elements. These filament proteins (e.g. SCP1 (mouse), Zip1p (yeast)) have central coiled-coil regions that function as protein interaction domains. Although SCP3 and therefore complete lateral element formation are unnecessary for a functional synaptonemal complex, condensin and cohesin do appear to be necessary for proper transverse filament attachment of the lateral elements.

Lateral elements are composed several proteins, including condensins and cohesins. The cohesins are meiosis-specific variants, with substitutions for the Scc1 and Scc3. Likewise, condensin subunits also have meiosis-specific alleles. In addition to the condensins and cohesins, which other than their meiotic-specific variants, are common chromosomal proteins, there are SC-specific proteins, including SCP2 and SCP3. Both are localized to condensed chromosomes in early meiosis, and SCP3 has been show by knockout analysis to be necessary for lateral element formation. However, it is not necessary for recombination.

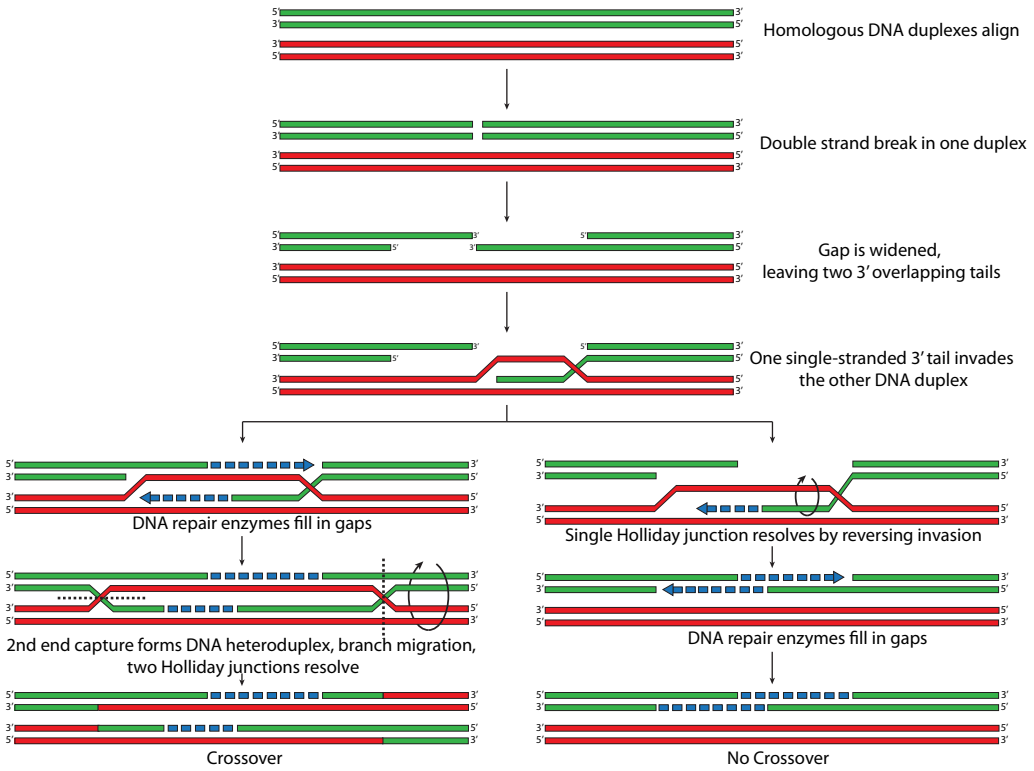


Figure 17. Recombination of homologous chromosomes.

Recombination may occur with or without the formation of double-strand breaks, and in fact, can occur without the formation of the synaptonemal complex, although the SC probably enhances the efficiency of recombination. In *S. pombe*, meiosis occurs without the formation of a synaptonemal complex, but there are small discontinuous structures somewhat similar to parts of the SC. In the fruit fly, *Drosophila melanogaster*, females undergo meiosis using a synaptonemal complex, but males do not undergo meiotic recombination, and their chromosomes do not form synaptonemal complexes. In most cases, recombination is preceded by the formation of recombination nodules, which are protein complexes that form at potential points for recombination. The best studied mechanism for meiotic recombination involves a double-stranded break of one of the chromosomes initiated by the meiosis-specific endonuclease, Spo11. The 5' ends (one in each direction) of this cut are degraded slightly to form 3' single-stranded overhangs. This leads to the formation of Holliday junctions with a strand from one chromosome acting as a template for a missing portion of the homologous cut chromosome. This may be resolved one of two ways, with or without a crossover, as illustrated (fig. 17).

The recombination is initiated in pachytene and completes in diplotene, at which time the synaptonemal complex breaks down. As the chromatids begin to separate, chiasmata become apparent at some of the recombination sites. As prophase completes, the chiasmata resolve from the center of the chromosomes to the ends.

As the cell goes from meiotic prophase I to meiotic metaphase I, another difference between mitosis and meiosis is revealed: the chromosomes line up at the metaphase plate as tetrads rather than as pairs. Because of this, when they pull apart in anaphase, sets of sister chromatids segregate to opposite poles. Of course, due to recombination, the sister chromatids are unlikely to still be identical.

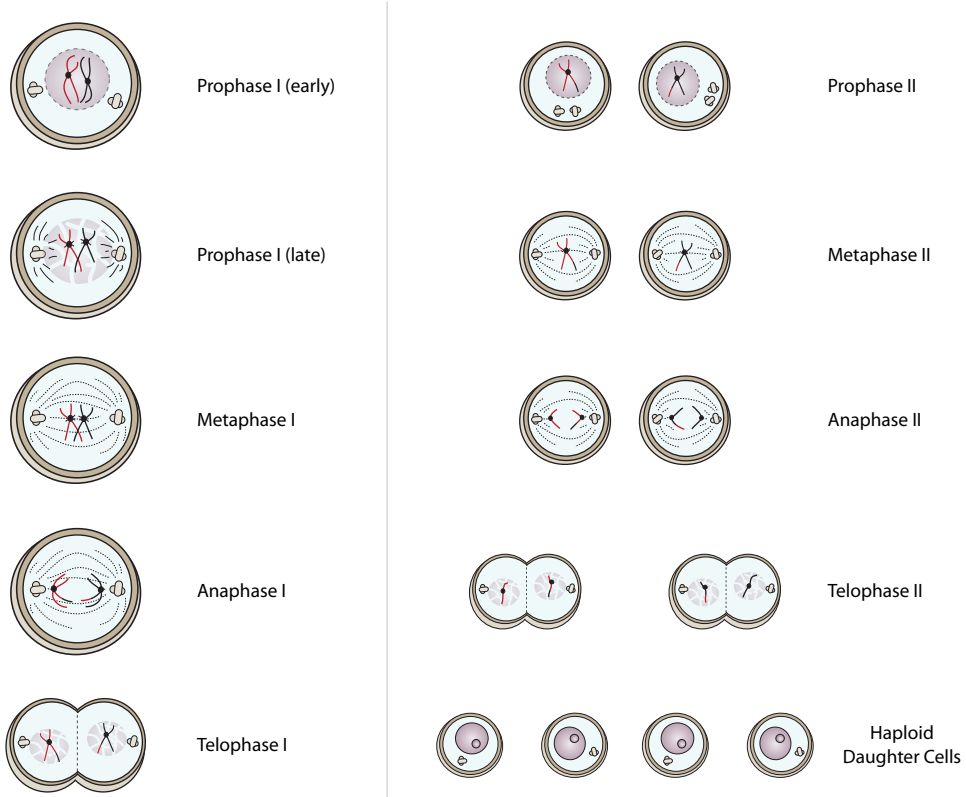


Figure 18. Meiosis generates 4 haploid daughter cells from one diploid precursor. To do so, it undergoes a two rounds of meiotic nuclear and cell division,

After a conventional anaphase and telophase, the cell splits, and immediately the daughter cells begin the second meiotic division (fig. 18, right side). In some cell types, chromosomes do not decondense in meiotic telophase I, but if they have, they re-condense

in meiotic prophase II. Prophase II proceeds similarly to *mitotic* prophase, in that there is no formation of synaptonemal complexes or recombination. At metaphase II, the sister chromatids line up along the metaphase plate just as in mitosis, although now there are only 2n chromosomes in the cell, while in mitosis there would have been 4n (because the DNA has replicated). Again, finishing the rest of the division almost exactly like mitosis, the sister chromatids pull apart in anaphase II, the nucleus reforms in telophase II, and the final cytokinesis generates a total of four cells from the original one that entered into meiosis, each containing 1n chromosomes.

Egg cells, as genetic and bulk material donors, need to be large but sperm cells, as genetic donors only, do not. The diagram below depicts the generation of the egg cells. Only one oocyte is generated from a meiotic event; the other three daughter cells are termed polar bodies, and contain so little cytoplasmic material that they are only viable for a short time. The asymmetric distribution of cytoplasm in the first meiotic division for oocytes is due to the position of the meiotic spindle in the periphery of the cell rather than centered. Since the center of the spindle determines the position of the contractile ring for cytokinesis, this leads to unevenly sized daughter cells.

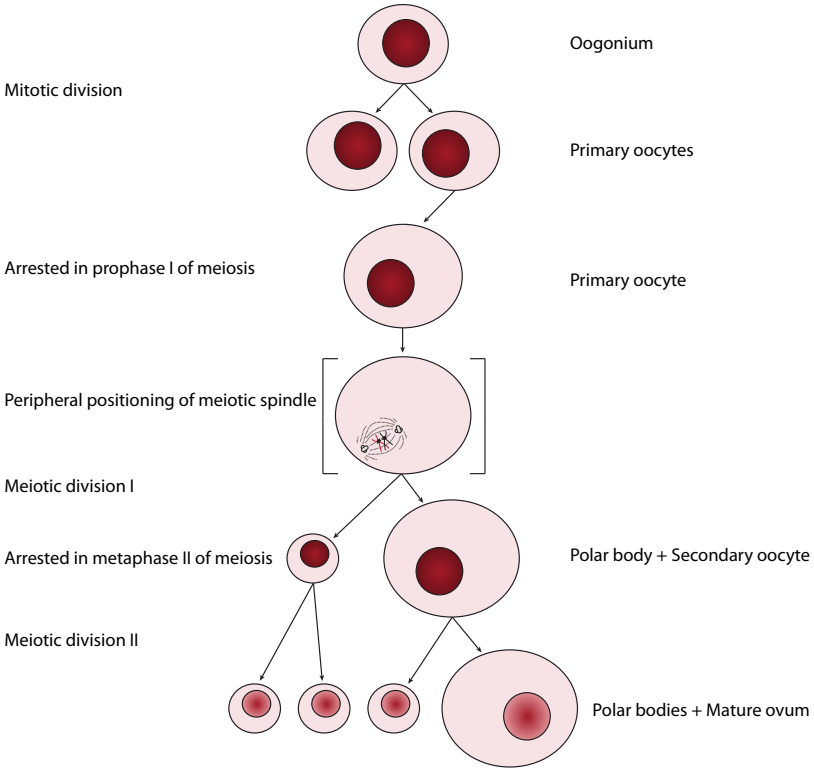


Figure 19. Oogenesis.

The generation of the very small sperm is a different mechanism altogether. In the meiotic steps of spermatogenesis, the cell divisions are equal, with the meiotic spindle aligned with the center of the cell, and the cells have equal amounts of cytoplasm, much like an average cell that has undergone mitosis. The streamlined, minimal-cytoplasm mature sperm is a product of post-meiotic differentiation, in which it gains the flagellar tail, and ejects most of its cytoplasmic material, keeping only some mitochondria to power the flagella, and an acrosomal vesicle, that contains the enzymes and other molecules needed to reach and fuse with (i.e. fertilize) an egg.

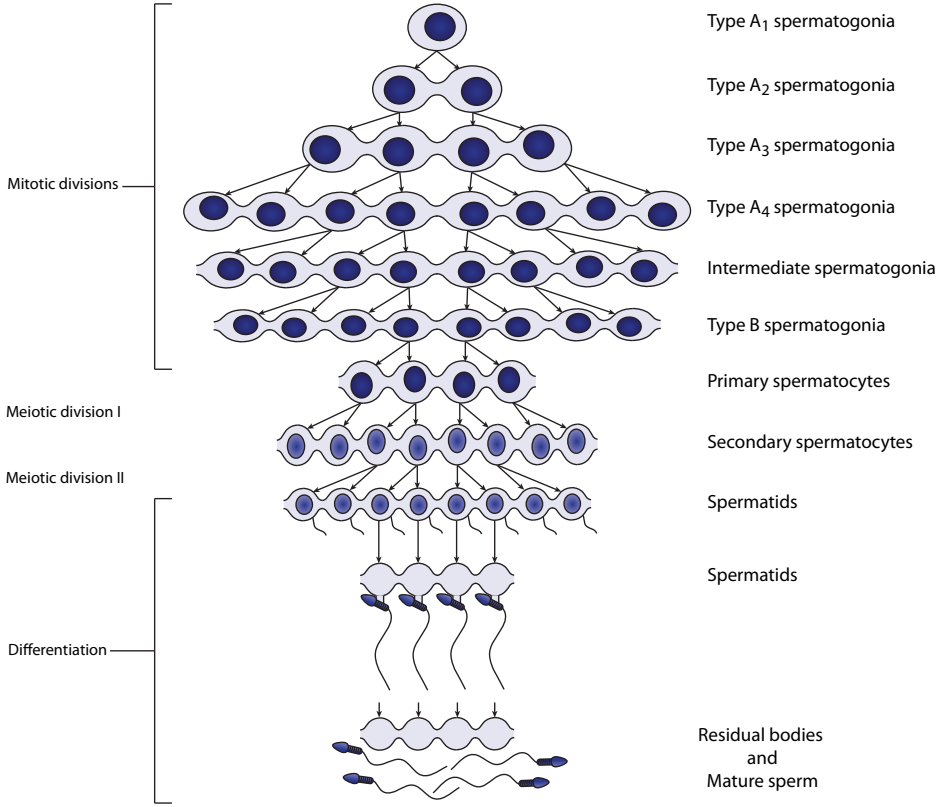


Figure 20. Spermatogenesis

Not all organisms reproduce with the human-like egg and sperm mechanism, i.e. gametic meiosis. As just described, in a gametic meiosis life cycle, meiosis generates haploid gametes, which then fuse/fertilize to become a diploid zygote. The zygote becomes a multicellular diploid organism, and once it reaches sexual maturity can make more haploid gametes via meiosis. The only multicellular state is diploid, and the gametes are haploid.

A common variation is sporic meiosis, used in all plants and many types of algae. In this usage, "spore" refers to eukaryotic spores, and not to bacterial endospores, which are simply dormant bacteria. Sporic meiosis does not directly produce gametes. Instead, meiosis produces haploid spores, which can develop by mitosis in haploid multicellular organisms. These organisms (termed gametophytes) can produce (still haploid) gametes by mitosis, that when fused/fertilized form a diploid zygote. This zygote can then develop into a diploid multicellular form called the sporophyte. Finally, the sporophyte is able to generate more spores by meiosis.

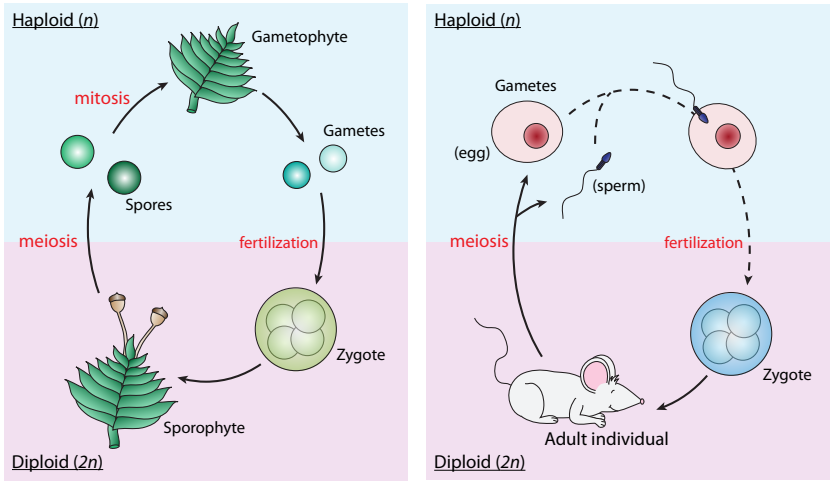


Figure 21. Gametic meiosis (left) and Sporic meiosis (right).

An example of this type of life-cycle and the role of meiosis is found in moss. What we think of as the body of the moss is actually a gametophyte, made up of haploid cells generated by mitotic division of a haploid spore. These gametophytes generate either sperm or eggs in specialized structures in their distal tips, and under the right conditions (e.g. rain) the sperm is carried to the eggs and fertilization occurs. The fertilized (diploid) egg now develops by mitotic division and differentiation into a sporophyte. In this case, the sporophyte is a specialized reproductive structure on the tip of the moss, and is also diploid. On the tip of the sporophyte is the sporangium, which is where meiosis takes place to generate haploid spores. The spores may then be dispersed (by wind or rain) and begin the cycle again by dividing and forming a new gametophyte.

Advanced Topics :

Viruses, Cancer, and the Immune System

Using this book: This book is designed to be used in both introductory and advanced cell biology courses. The primary text is generally on the left side of the vertical divider, and printed in black. Details that are usually left to an advanced course are printed in blue and found on the right side of the divider. Finally, additional biomedically relevant information can be found in red print on either side of the divider.

At this point, you should be fairly comfortable with the basic concepts of cell biology. The purpose of this chapter is to build on that basic knowledge and put it together into more complex systems. In addition, we will introduce some more advanced variations on some of the mechanisms and structures that were discussed in earlier chapters. The three topics, viruses, cancer, and immunity, are not only relevant as current news topics, but relate to one another through multiple pathways, which is why they are lumped together.

Viruses

Though a virus has both genetic material and protein components, it is not a living organism. It does not contain the capability of self-replication, and is completely reliant on the cellular biochemistry of whatever host cell it has infected. The minimal definition of a virus is a nucleic acid genome inside of a protein shell, or capsid. There are variations of this, such as virions (infectious viral unit) that have a membrane coat outside of the capsid, or some that have enzymes inside of the capsid alongside the genome. Again, none of these viral variations are able to fully replicate without cellular machinery. The cells that viruses can infect range across most living organisms. There are viruses specific for humans, some that only infect particular animals, some that infect plants, and even viruses that use bacteria as hosts. In current media reports of viral outbreaks in recent years, HIV, avian flu, swine flu, much is made of the origin of the virus with respect to its host/target organisms. However, most viruses are very specific about the cells that they infect. The narrow host range may be not only to particular species, but particular cell types within a particular species. Viruses with a broad host range are relatively rare. However, this does not preclude the possibility of new strains of virus evolving with different host ranges from their ancestral virii.

Viruses may have either RNA or DNA genomes, that may be linear or circular, and single or double -stranded. There are fewer variations of capsid structure. In general, capsids fall into two categories: helical and icosahedral. Helical capsids are actually made up of globular subunits that associate into a helical cylinder, with the genome lying inside

Viroids are also infectious non-living particles, but they are only genetic material, RNA, and have no protein capsid. To date, they are only known to infect plant hosts, and apparently spread by direct or extremely close contact with an infected plant. These infectious pathogenic RNA molecules are single-stranded and circular, and relatively small, roughly 200-400 nucleotides.

an interior groove of the helix (fig. 1c). The icosahedral capsids are also made up of many subunits that together form an approximately 20-sided polygon made from sides that are equilateral (or nearly so) triangles. If you play Dungeons and Dragons™ or know someone who does, then you have probably seen dice (a "d20") of this shape. Of course with capsids, but not dice, there can be some variation in the number of sides, the shape of the triangles, and the number of subunits that make up each face.

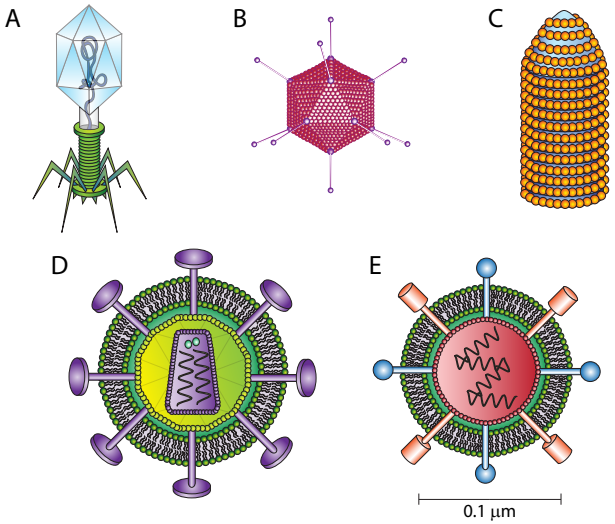


Figure 1. Viruses. (A) a T-4 bacteriophage, (B) adenovirus, (C) tobacco mosaic virus, (D) human immunodeficiency virus, (E) influenza virus.

External to the capsid, some viruses also have a membrane coat (viral *envelope*). As will be more clearly explained soon, this phospholipid bilayer comes from viruses that exit a host cell by exocytosis. Because it came from a host cell, the membrane can be used as a ruse by the virus to fool other potential host cells into misrecognizing the virus as a normal cell or cell debris, based on the receptors that recognize cellular proteins on the membrane. It may then be taken into the cell by receptor-mediated endocytosis in a mistaken attempt to recycle old cell debris, where it can proceed to infect the overgenerous host.

There are two classification systems for viruses, the International Committee on Taxonomy of Viruses (ICTV) has a Linnaean-like taxonomic system based on shared structural or biochemical properties (but not host specificity). Another system, also in use, is the Baltimore classification, in which viruses are classified into seven categories by the mechanism of mRNA production. That is, type I are the dsDNA viruses that make mRNA the "normal" way by direct transcription of the genome, type II are ssDNA viruses that

Viruses in all categories (ICTV class or Baltimore type) that can cause human disease. Some of the major ones are listed here.

Virus	Class	Disease
Adenovirus	Adeno	Febrile respiratory disease
		Pharyngoconjunctival fever
Epstein-Barr	Herpes	Infectious mononucleosis
Hepatitis A	Picorna	Acute hepatitis
Hepatitis B	Hepadna	Acute/chronic hepatitis
		Hepatic cirrhosis
		Hepatocellular carcinoma
Hepatitis C	Flavi	similar to Hepatitis B
Herpes Simplex		Cold sores, pharyngitis
Type 1	Herpes	Gingivostomatitis
Herpes Simplex		Aseptic meningitis
Type 2	Herpes	Genital herpes
HIV	Retro	AIDS
Influenza	Orthomyxo	Influenza
Measles	Paramyxo	Measles
Mumps	Paramyxo	Mumps
HPV	Papilloma	Cervical cancer
		Genital warts
Poliovirus	Picorna	Poliomyelitis
Rabies	Rhabdo	Rabies
Rubella	Toga	German measles

must first make a complementary strand to become dsDNA before transcription, type VI are ssRNA viruses that use reverse transcriptase (detailed later in this section) to first convert the RNA to a DNA intermediate before transcription, and so on.

Lytic "life" cycle of viruses

Viruses can interact with their hosts in two distinct ways: the lytic pathway and the lysogenic pathway. Some viruses are able to switch between the two pathways while others only use one. The distinguishing characteristic of the lytic life cycle is catastrophic death of the host cell by lysis and simultaneous release of viral particles. In figure 2, the stages of the lytic pathway are depicted. In this case, a T4 bacteriophage (the term "phage" is used for bacterial viruses) is used as an example. In step 1, the virus attaches to the cell wall. In step 2, the virus injects its genetic material (dsDNA) into the cytoplasm of the bacteria. In step 3, the viral DNA is being replicated and the genes on the viral DNA are being transcribed and translated into viral proteins. Expression from the host genomic DNA is arrested. In step 4, viruses are assembled from the proteins and DNA. And finally, once the viral factory has used up the cell's energy and material resources in making more viruses, it performs a final coup de grace, as cell is destroyed to free the viruses to exit and find more host cells. The T4 phage used in this example only undergoes this pathway and not the lysogenic pathway.

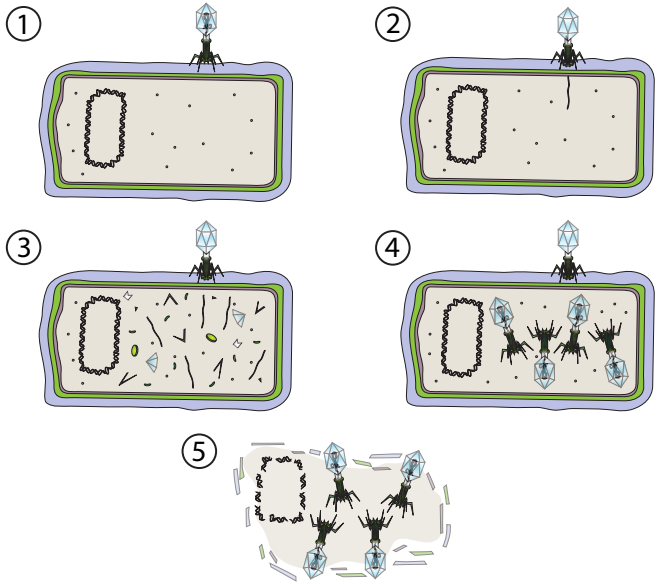


Figure 2. The Lytic Pathway

In eukaryotes, the mechanism is slightly more complicated by the nucleus. The DNA is transported into the nucleus, where the transcription and replication take place. Although the viral mRNA is transported out to the cytoplasm for translation as expected, the resulting capsid proteins are then imported back into the nucleus, where the virion particles are assembled. Lytic plant and animal viruses with RNA genomes can bypass the nucleus altogether, and the genome replication, protein synthesis, and particle assembly all occur in the cytoplasm.

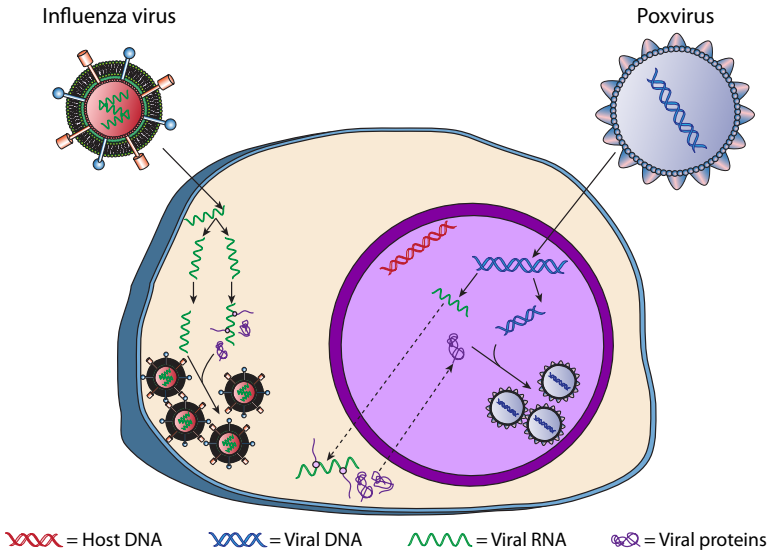


Figure 3. Lytic viruses in eukaryotes: an RNA virus (influenza) replicates completely in the cytoplasm, while the DNA virus (poxvirus) uses both cytoplasm and nucleus.

The lytic pathway can produce a huge number of viral particles between infection and lysis, as many as several tens of thousands, for example from a rabies-infected cell. Therefore, this pathway is well suited for conditions in which potential host cells are plentiful. On the other hand, this is a waste of resources if there are relatively few potential hosts. Imagine a few bacteria that have floated off from the colony: if a phage infected a bacteria in the main colony, commandeering the bacteria to create thousands of viral particles, most of those particles would infect new hosts and make many thousands more soldiers in this viral army. But if the virus infected one of the breakaway bacteria, then once it killed its host by lysis, the viral particles would have few, if any, other potential hosts, and eventually all the viral particles just break down from various environmental conditions. What would a better survival strategy for the virus in such a situation?

The Lysogenic Pathway

A better option for some bacterial viruses is called the lysogenic pathway. The bacteriophage that have this option, as well as a lytic pathway, are known as temperate phage. In this pathway, the virus goes into dormancy by integrating into the host genome, and remaining transcriptionally quiescent until environmental conditions change and reflect a likelihood of more host cells to infect (fig. 4). Lambda (λ) is an example of a

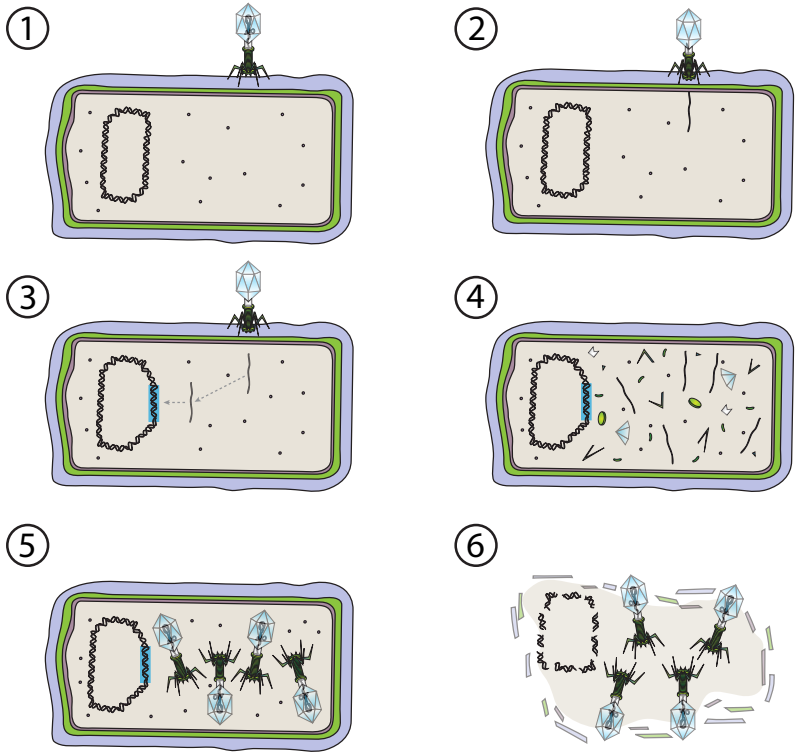


Figure 4. The lysogenic pathway.

temperate bacteriophage. The initial stages of infection and genome injection are the same as the lytic cycle, but under conditions that encourage lysogeny, the viral genome is integrated into the host genome in step 3. In λ integration into E. coli, this occurs by reciprocal recombination at a 15-base pair sequence known as the att λ site and is facilitated by the Int gene product. As long as the environmental conditions are not conducive to bacterial reproduction (and thus limited number of possible host cells), the viral genome remains mostly hidden and inactive. The only significant exception is a gene encoding a λ repressor that prevents the next step and keeps the virus dormant.

That next step is the excision of the l phage DNA from the host chromosome, and subsequent replication and transcription of the viral DNA (fig. 4, Step 4). Then, like before, the final steps are assembly and accumulation of virions, and eventual breakdown of cellular structure and release of the viral particles.

Although it is not referred to as lysogeny, some animal viruses can behave similarly. The most prominent example is the Baltimore Class VI viruses - commonly known as retroviruses, one of which is HIV. The path of a retrovirus through a eukaryotic host cell is depicted below (fig. 5). HIV has an envelope, which is studded with transmembrane proteins that are recognized by the host cell, binding the virus to the cell surface and initiating receptor-mediated endocytosis (1). After the endocytosis, the membrane envelope of the virion and the vesicular membrane fuse to release the capsid and its contents (2). After the capsid dissociates in the cytoplasm, the two strands of viral RNA are released along with a special polymerase: reverse transcriptase, which reads an RNA template and synthesizes DNA. Reverse transcriptase also uses that new DNA to synthesize a complementary DNA strand so that it eventually produces a double-stranded DNA version of the viral genome (3). This viral dsDNA is transported into the nucleus where it integrates into the host genome using another viral protein, integrase

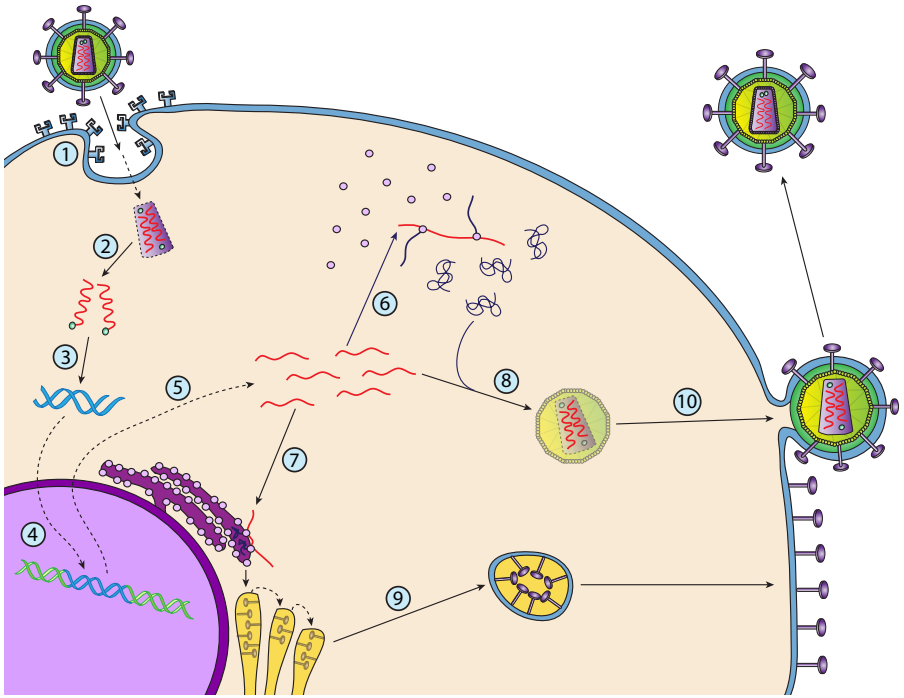


Figure 5. Infection and reproduction pathway of retrovirus in a eukaryotic host cell.

In order for packaging into the tight space constraints afforded by capsids, viral genomes must be highly economical. For example, the HIV genome (fig. 6) has several genes that overlap.

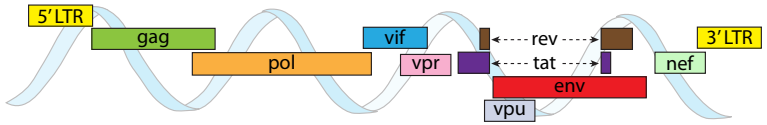


Figure 6. The HIV genome.

Or, in the case of curtoviruses, ssDNA plant viruses (e.g. beet curly top virus), the genome not only has overlapping genes, it is even bi-directional (fig. 7) encoding gene products in both strands of DNA after the ssDNA has been converted to dsDNA.

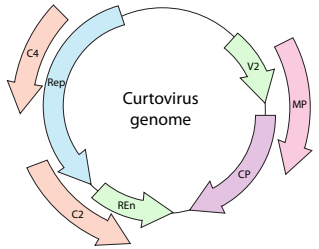


Figure 7. A curtovirus genome.

Given the need for economy, what genes are found in viruses? One of the most studied viral genomes, bacteriophage λ, contains genes encoding five transcriptional control proteins (which ones are expressed depends on whether the phage is in a lysogenic or lytic mode), a binding protein that controls degradation of a transcriptional activator, 17 capsid proteins, an excisionase that controls excision and insertion of the phage genome in the host genome, an integration protein that inserts the phage genome into the host's, and 3 genes participating in lysis of the host cell.

The HIV genome depicted above is much smaller than λ, at around 9 kilobases compared to 48 kb, but again, the theme is to use cellular proteins when possible, and encode viral genes if necessary. So, *gag* encodes capsid proteins, *pol* encodes reverse transcriptase, integrase, and HIV protease (which cleaves the gag and pol gene products into their functional proteins), *vif* acts against a common host cell antiviral enzyme, *vpr* regulates nuclear import, *tat* strongly increases transcription of HIV genes, *rev* exports viral RNA from the nucleus, *vpu* is needed for budding of particles from the host, *env* encodes viral envelope glycoproteins, and *nef* promotes survival of infected cells. The LTR regions are very strong promoters to drive high expression of these genes.

(4). The integrated viral DNA is called a provirus. The provirus can lay dormant, but if it is activated, then it is transcribed and the resulting viral RNA is transported out of the nucleus (5). Some of the viral RNA encodes enzymes like reverse transcriptase and integrase, or capsid proteins, all of which are made in the cytoplasm (6), but some encode membrane bound glycoproteins, which are translocated into the ER (7) and eventually processed through the Golgi and incorporated into the plasma membrane (9). Once the virion has been assembled (8), it binds to the viral transmembrane proteins, nucleating an exocytic "vesicle" (10) which is the virion complete with viral envelope.

In considering viruses with respect to the rest of this integrative chapter, there are two overriding ideas to keep in mind. First, viral survival is based on numbers: it needs to make huge numbers of its components to cast as wide a net for new host cells as possible. To do this, viral promoters are usually much stronger than host cell promoters, simultaneously driving more viral gene expression while preventing host gene expression (by dedicating cellular resources to virus production). Second, because of fast generation times, the rate of viral mutation and evolution is far faster than normal eukaryotic genomes. In addition, if the virus uses its own polymerase (such as reverse transcriptase or RNA replicase), the mutation rate rises even more because there is no error-checking by viral polymerases.

Cancer

Cancer encompasses a set of genetic diseases that lead to uncontrolled cell proliferation in multicellular organisms. The discussion of cancer also happens to be useful in a cell biology course, because it ties together many of the concepts that you just spent most of the semester learning. Although it can be caused in part by an outside agent, the development of cancer is essentially a series of uncorrected mistakes by a cell's regular processes. It can strike plants as well as animals, and because of intense re-

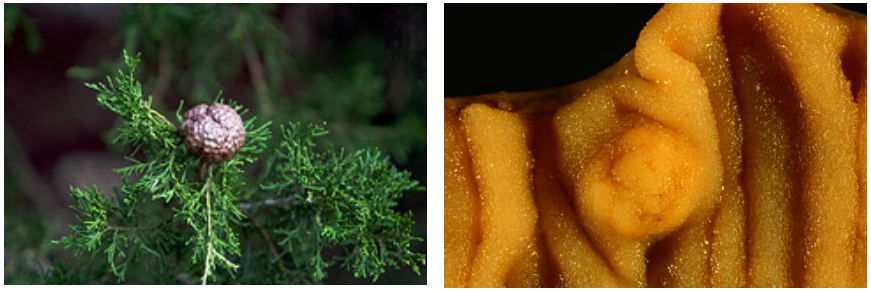


Figure 9. (left) A tumor on a cypress branch. (right) A tumor of the small intestine. Cypress tumor photo by W. Calder, cc licensed 2009. Small bowel tumor by E. Uthman, public domain 1999.

Recent structural examination of the HIV genome suggests that the structure of HIV RNA itself may play a significant role in its propagation inside of host cells. Figure 8, from Watts et al, *Nature* **460**:711-716, 2009, shows a predicted secondary structure of the genome. The authors suggest that the RNA structure actually may interact with ribosomal elongation to control the folding of the viral proteins. They also postulate the extension of this argument to include important genetic information encoded not just in the nucleotide sequence, but the secondary structure and tertiary structure of any RNA virus.

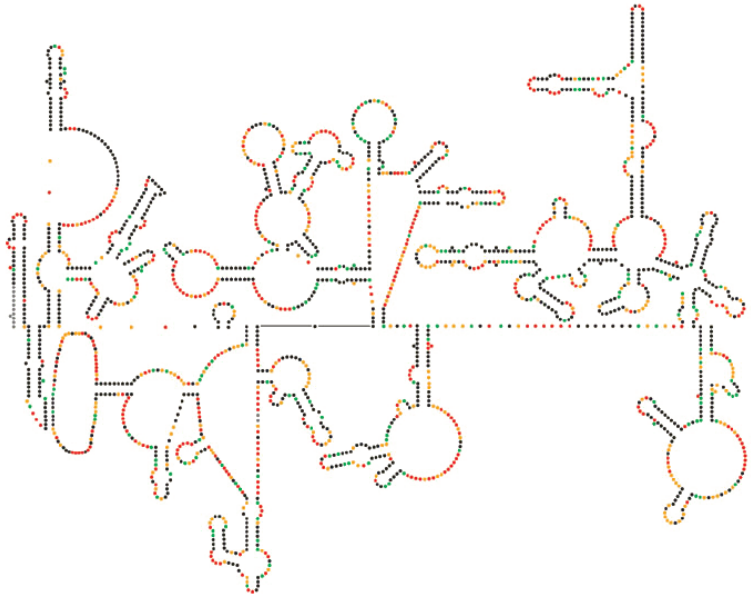


Figure 8. Secondary structure of HIV-1. (reprinted by permission from Macmillan Publishers Ltd: Nature 460:697, 2009)

search and subsequent deeper understanding of the cellular events that lead to cancer, it can now be treated in humans with some degree of success, depending on the type, location, and progression of the tumor.

Abnormal replication of a cell generally leads to the formation of a tumor, which is simply a solid mass of abnormally growing cells, usually clonal colonies of one or a few original tumorigenic cells. However, a tumor is not necessarily cancerous. A benign tumor is one that is ensconced within an extracellular matrix sheath, does not spread beyond that sheath, and whose growth is slow or limited. In contrast, a cancerous or malignant tumor grows quickly due to uncontrolled proliferation, expands significantly beyond its original boundaries, invading new tissue, and can metastasize, spreading through the circulatory system. Once this happens, not only is it no longer possible to remove all of the cancerous cells by surgical excision of the primary tumor, it is also nearly impossible to know how many secondary tumors have formed or where they formed, since the metastatic cancer cells in the bloodstream may theoretically exit almost anywhere. However, in reality, certain tumors metastasize preferentially to particular target tissues/organs, presumably based on molecular markers on the surface of the cells or in the extracellular matrix. Metastasis is considered the greatest medical problem with respect to cancer treatment. If cancer is detected after metastasis has occurred, the chances of survival drop dramatically.

At the cellular level, cancerous cells differ from normal cells in a number of important ways. Normal cells are regulated by the cells around them, and by adulthood, most cells are inhibited from proliferation by contact with their neighboring cells. In vitro, this can be demonstrated by the observation that non-cancerous proliferative cells such as epithelial cells can proliferate until the culture dish bottom is completely covered

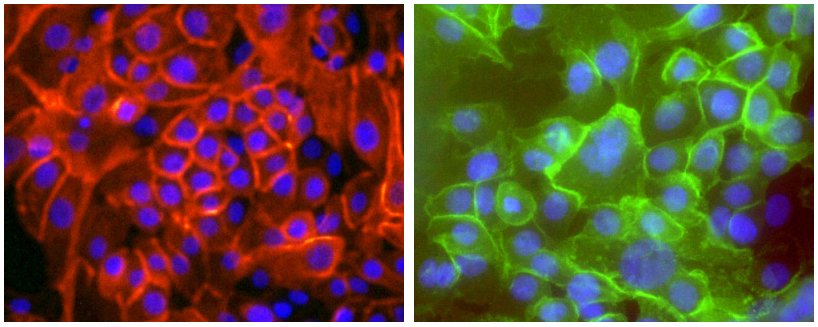


Figure 10. Normal human breast cells in culture at left. At right, similar cultured cells that have been transformed (i.e. they are now cancerous). Note the irregularity of both cell and nuclear morphology. Membranes are arbitrarily stained in different colors; chromosomes are stained blue in both panels. Photos from Ince et al, Cancer Cell 12:160-170, 2007.

(confluence), but once that happens, proliferation stops. This phenomenon is known as contact inhibition. If cancerous cells are allowed to proliferate in culture, they do not stop after the surface is covered, and instead can mound up on one another. The cell surface and internal cellular organization of cancer cells is often disorganized in comparison to normal cells. Finally, cancer cells usually appear de-differentiated in comparison to their original cell type. If the original cell type was a flat cell, the cancerous cell would be more rounded and three-dimensional. This is an expected consequence of becoming a cancerous cell. Not only is proliferation deregulated, cell surface protein expression is altered to promote metastasis.

Cancer is considered a *genetic* disease because it is caused by alterations to the DNA. However, it is rarely an *inherited* disease. An inherited disease would mean a disease that can be passed from one generation to the next, implying that the disease-causing DNA mutation is found in the gametes (sperm or egg) of the stricken adult. Most cancers are due to spontaneously arising mutation in the DNA of one or a few somatic cells, and not a systemic aberration. Spontaneous mutation in the germ cells are possible, but most potentially cancer-causing ones lead to non-viable offspring. So, although it is exceedingly rare for cancer to be inherited, however, it is much more common to inherit a predisposition or increased chance of developing a cancer.

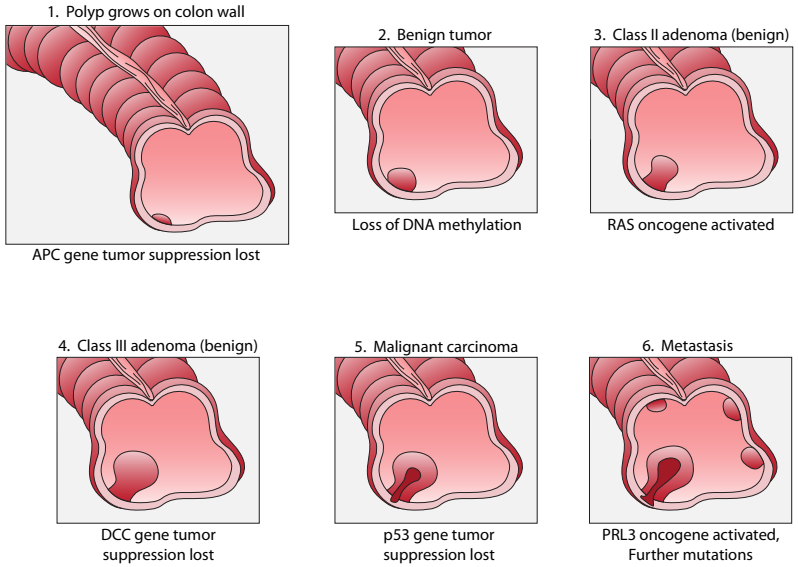


Figure 11. Development of colon cancer takes time and multiple mutations.

Differentiation is a key part of normal metazoan development. All cells come from the fertilized egg, and even after several divisions, the cells are very similar. Eventually, though, they begin to specialize for their particular physiological functions, whether as lung cells, brain cells, or bone cells, and that process of specialization is differentiation. In cancer cells, this process is partially reversed, as the cell reverts to a less specialized, more primitive state.

An individual cancer-causing mutation generally creates a problem that can be corrected by some other cellular mechanism. Therefore, development of cancer comes about through the accumulation of multiple mutations and not the acquisition of just one. The best studied example of this gradual development of cancer is colon cancer (fig.11). There is a fairly characteristic progression of mutations in the genes APC, RAS, DCC, TP53, and PRL3. Note that the progression depicted here is not inevitable: the presence of polyps does not lead invariably to colon cancer. Furthermore, intervention can be highly successful if it occurs early in the progression, so oncologists need to consider a range of risk factors in weighing the cost and benefits of medical intervention. RAS and PRL3 are oncogenes, while APC, TP53, and DCC are tumor suppressor genes.

Oncogenes

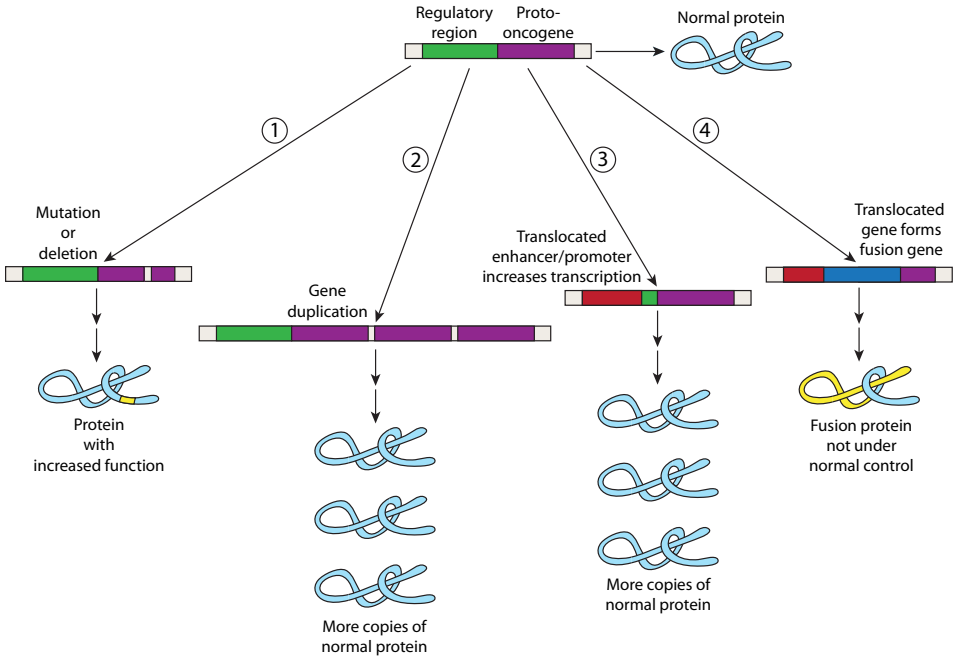


Figure 12. Conversion of protooncogenes to oncogenes. (1) Due to mutation of the coding region, the protein has higher physiological activity. (2) Gene duplication leads to multiple copies of the gene each expressed normally, making many more copies of the protein than normal. (3) Mutation of the regulatory region or translocation of a stronger enhancer or promoter to the protooncogene can lead to enhance transcription, and therefore more protein. (4) Translocation of another gene inline with part of the coding region, can put the activity of the protooncogene under the control of modifiers of the translocated gene, and thus lead to overactivity.

Oncogenes are generally dominant gain-of-function mutations of normal cellular genes called protooncogenes. These protooncogenes are themselves positive regulators of the cell cycle, but they are regulated by other factors, either extracellular signals or intracellular mechanisms. Mutations that turn them into oncogenes specifically remove all or some of this regulation. They thus become overactive, and try to push the cell cycle forward leading to increased proliferation. These mutations can also be classified into a few general mechanistic categories. These (fig. 12) are mutations to the coding region that increase physiological activity, gene duplications resulting in more copies of the gene at the DNA level and thus more at the protein level, mutations to the regulatory region of the gene or that alter regulation of gene expression, thus increasing copy number of the protein, and finally, translocations that replace part of the coding region, resulting in a chimeric protein whose activity may be under a different control scheme than normal.

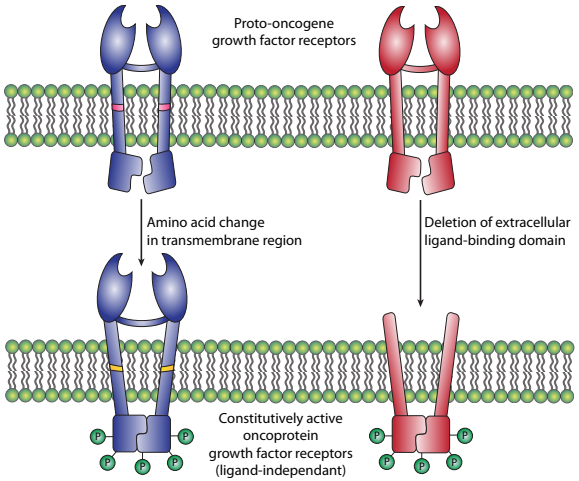


Figure 13. Conversion of a mitogen receptor protooncogene into an oncogene by point mutation leading to amino acid change in the transmembrane region (left) or by truncation of ligand-binding domain.

Examples of two types of mutations are illustrated to the right (fig. 13) with a mitogen receptor as the protooncogene. In the first case, the transmembrane portion of the receptor has been mutated, causing an amino acid change that alters the conformation not just of the transmembrane region, but of the cytoplasmic kinase domain, which becomes constitutively active, regardless of whether a ligand has bound outside or not. In the second case, the entire extracellular domain has been removed due to a mutation of an amino acid codon into a stop codon or translocation, and the resulting receptor is always active, also independent of ligand binding.

Some kinds of retroviral infection can accomplish the conversion of a protooncogene to an oncogene by inserting viral DNA near the promoter region of the protooncogene. Because the viral promoters tend to be very strong, they can induce overexpression of the protooncogene product. In avian species, avian leukosis virus is known to cause tumors by insertion near the c-myc oncogene, while in humans, another retrovirus, HTLV (human T-lymphotropic virus), can cause acute disease (tropical spastic paraparesis), but may also cause T-cell leukemia and lymphoma.

What functions are characteristic of protooncogenes? Mitogen receptors, as already described above, and exemplified by the receptor tyrosine kinases EGFR (epidermal growth factor receptor), VEGFR (vascular endothelial growth factor receptor), RON (recepteur d'origine nantais, a macrophage stimulating protein receptor), and ErbB2 (also HER2/neu, another human EGF receptor). Growth factors themselves may also be protooncogenes, such as FGF-5, one of several oncogenes in the fibroblast growth factor family, or c-sis, an oncogenic form of PDGF (platelet-derived growth factor). Signal cascade proteins, often either tyrosine or serine/threonine kinases or other regulatory enzymes, are a large group of protooncogenes (e.g. Src family tyrosine kinases, BTK family tyrosine kinases, cyclin-dependent Ser/Thr kinases, Ras-family small GTPases). Finally, various transcription factors (e.g. Ets, Myc, E2F families), can effectively be mutated into oncogenes.

Tumor Suppressor Genes

Tumor suppressor genes normally do what would be expected from their name. Whereas the oncogenes mostly drive the cell cycle forward, the tumor suppressor genes' primary functions are to temporarily stall the cell cycle so that DNA repair mechanisms can have time to work. However, if repair is unsuccessful after a few attempts, the tumor suppressor gene product may then trigger apoptosis rather than allow a damaged cell to replicate and potentially create another genetically damaged cell. Thus, the presence of an oncogene in a cell will not necessarily lead to development of cancer because a functioning tumor suppressor gene might prevent the cell from replicating. Equally, if a tumor suppressor gene is knocked out but there is no oncogene present, then the cell is unlikely to be immediately cancerous because although a cellular "emergency brake" is nonfunctional, if there is nothing to drive the cell through its cycle any faster or more frequently than usual, then the "brake" is never needed anyway.

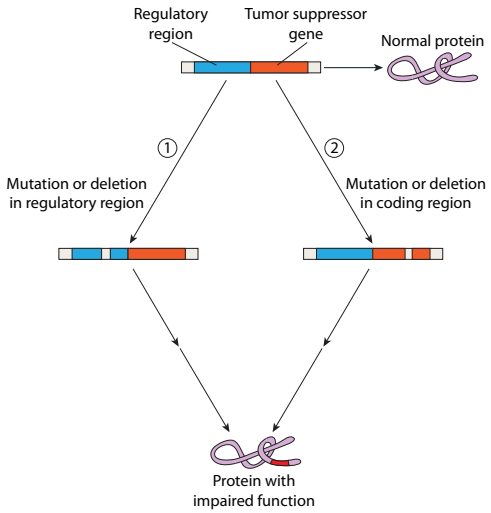


Figure 14. Tumor suppressor gene mutations can lead to cancer.

Like oncogenes, tumor suppressor genes can work (or not work, as would be the case in cancer) in several ways. Here is an example with the breast cancer-associated genes, BRCA1 and BRCA2. These gene products are involved in DNA repair (chapter 7). When BRCA1 or BRCA2 is knocked out, the cell loses its ability to use that DNA repair pathway. There are other repair pathways, and even if there weren't there may not be any serious lesions to the DNA, so the cell could behave normally for the time being. What is important from a cancer standpoint, is that each safety/repair mechanism that is lost increases the likelihood that an additional mutation may cause the cell to become cancerous.

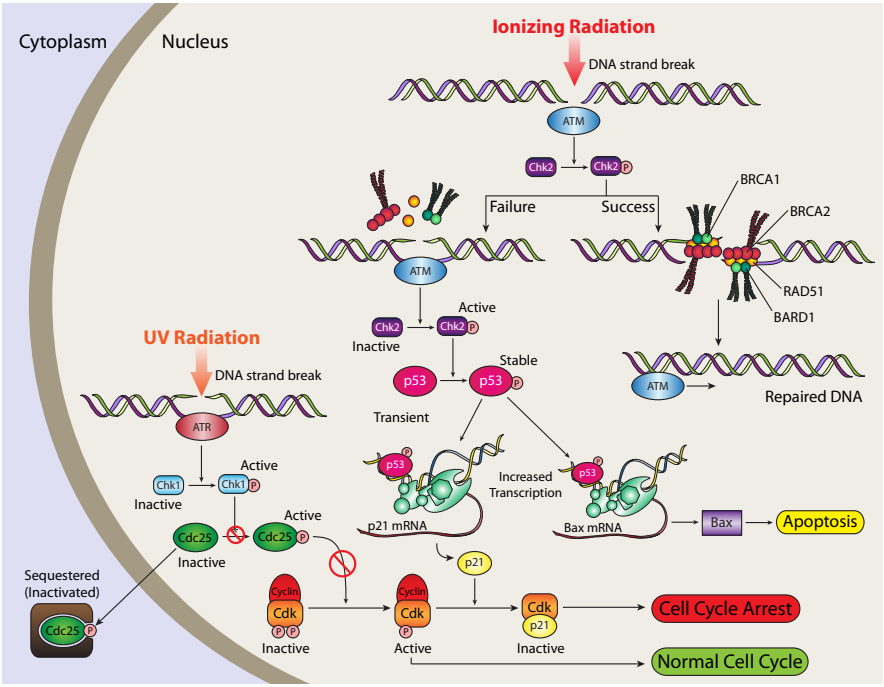


Figure 15. Cell cycle arrest due to DNA damage. ATM detects the double strand break, and activates Chk2 and BRCA1. Chk2 also activates BRCA1, which with BRCA2 forms a repair complex. However, if BRCA1 is not immediately available, the cell needs to go into a holding pattern until one becomes available. Therefore, Chk2 activates p53, which induces transcription of p21, which binds to cdk, preventing association with cyclin, and thus preventing cell cycle progress. If this continues for long, some of the p53 activates transcription of Bax, which will induce apoptosis to kill off a cell with damaged DNA. When p53 is hit with a loss of function mutation, the cell does not die, and it attempts to replicate even with damaged DNA, which may lead to more mutations in the subsequent generation, if it is successful in reproduction. Without p53, the accumulation of errors in successive generations increases. The mechanism of buying time for the cell to make repairs is not limited to the ATM-BRCA situation. The left side of the figure shows another response to DNA damage that leads to cell cycle arrest.

It should be clear now how recessive loss-of-function mutations in a tumor suppressor gene can lead to an inherited predisposition to cancer. As diploid organisms, we have two copies of each gene in our cells, so losing one to mutation does not wipe out the protective function. Thus, if nothing happens to the other one, then the cell is fine. It is just a question of probability. Losing the function of one is a very low probability event, but the probability of losing both copies is extremely small. Thus, even though it is "only 1 step" on the way to losing the protection of this particular tumor suppressing function, it is a very large difference in probabilities. Of course, keep in mind that even complete loss of a single tumor suppressor gene is usually not enough to lead immediately to cancer, and still other mutations must occur to take advantage of the weakened cell defenses and push it towards a cancerous state.

Human Cancers

Although some oncogenes and tumor suppressor genes have a restricted distribution that hints at likely tumor locations, many of the genes are widespread and even ubiquitous. It is presently unclear, therefore, why certain types of cancer are linked to particular mutated genes, but there are a number of strongly correlated cases.

Retinoblastoma is a cancer of the eye that usually strikes at a relatively young age. It has been linked to the RB gene, which encodes a repressor of E2F, a transcription factor that would normally turn on genes needed for S phase progression. Only 10% of individuals who inherit the RB loss-of-function mutation escape the development of the cancer. It also turns out that people with the RB mutation have a higher incidence of developing other tumors as well, although generally later in life. Perhaps the higher rate of damage to retinal cells (due to light exposure) leads to greater susceptibility.

Breast cancer is another disease that has strong links to mutations in certain genes. Loss of function mutations to the BRCA1 gene encoding a DNA repair protein lead to a five-fold higher risk of developing breast cancer in a woman's lifetime. Although mutations to other tumor suppressors (including p53, PTEN, CHEK2, ATM) most hereditary breast cancers have a link to BRCA1 or BRCA2 mutation. On the oncogene side, breast cancer tumors consistently show expression of CYCD1 (a cyclin) mutations, and depending on the type of tumor, HER2/Neu may be linked as well.

Lung cancers are among the most common - the second highest in men (prostate is higher) and women (breast is higher) alike, and make up approximately 1 in 3 cancer deaths annually. Several oncogenes of the myc family: N-myc, L-myc, and c-myc, as well as H-ras have been linked to various lung cancers. Loss of p53 and RB are also associ-

Cancers are classified by the tissue type in which the tumors first arise. Thus, *carcinomas,* which are the most common type (~85% of human cancers), come from epithelial cells arising from either the embryonic ectoderm (skin and nerve cells) or endoderm (gut lining). *Leukemias* (~4%) arise from white blood cells. *Lymphomas* (~5%) reflect aberrant growth of lymphocytes in spleen or lymph nodes. *Sarcomas* (~2%) arise from connective tissue of mesodermal origin, such as bone cancers.

ated with the development of lung cancers, and perhaps not coincidentally, tobacco smoking is associated with p53 mutations. Interestingly, despite being so common, so far, there have been no particular oncogenes associated with prostate cancer, nor any hint of prostate-specific tumor suppressor susceptibilities.

Metastasis

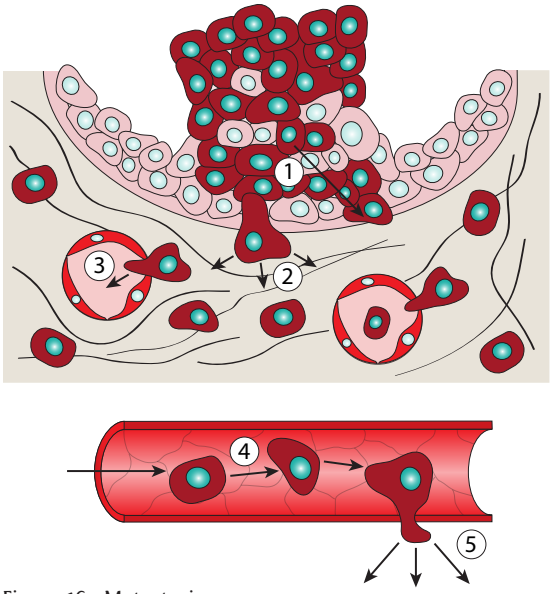


Figure 16. Metastasis.

The onset of metastasis signals a drastic change in the prognosis of a cancer patient. While pre-metastatic tumors can certainly be dangerous or painful, treatment can be fairly localized, e.g. surgical excision and directed radiation therapy. Once the tumor metastasizes it must be treated systemically due to the potential for secondary tumors literally anywhere in the body. This presents a problem because the tumor cells are derived from the body's cells and are mostly indistinguishable by the body's immune system. The primary mechanism for anti-cancer drugs is to target fast proliferation, since most cancer cells proliferate much faster than most normal cells, but this still kills off some of the body's naturally fast-proliferating cells, such as the epithelial cells lining the gut. More recently, other approaches to anti-cancer drug treatments have been developed; most notably, anti-angiogenesis drugs to starve tumors by preventing them from developing or recruiting new blood vessels. As tumors grow, the ability to absorb nutrients from the environment decreases for the innermost cells of a solid tumor.

Metastasis (fig. 16) starts with downregulation of cell-cell adhesions (1). This may include inside-out signaling to integrin receptors, or downregulation of cadherin expression, and other methods for allowing the cell to separate from the rest of the tumor. Non-metastatic tumors are surrounded by a capsule of extracellular matrix that contains the tumor in its location. To escape this capsule, the metastasizing cell must secrete proteases (usually metalloproteases) that can break down the ECM proteins (2). Once out into the looser connective mesenchymal tissue, the metastatic cell increases

its locomotive activity and heads for a blood vessel. Intravasation (3) into a small, low-flow blood vessel allows the cell to be carried to nearly any destination in the body by the circulatory system (4). At some point, the metastatic cell will attach to the interior wall of a blood vessel, and exit the circulation (5). The molecules and situations that determine the point of exit are not clear yet, although there are clearly preferred sites of metastasis for some types of tumors. Presumably, there is specific recognition and adhesion occurring based on cell adhesion molecule expression on tumor cell and target tissue surfaces.

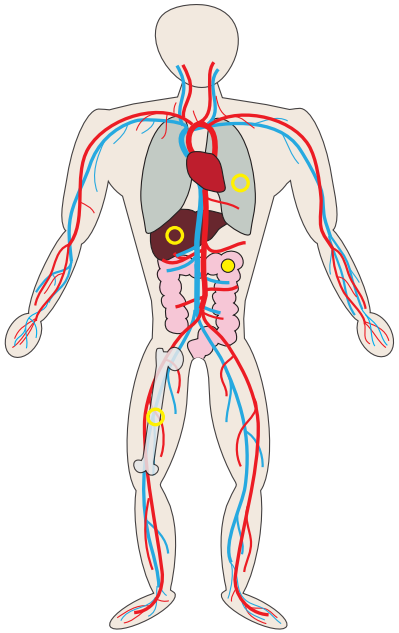


Figure 17. Common sites (open yellow circles) for the metastasis of colon cancers (filled yellow circle).

The Immune System

Immunology is a full semester course at most universities, so this section will only touch on a few basic concepts that should be easily accessible to the student who has nearly completed the cell biology course. At its core, immunology is about adaptation. That is, since an animal has no preconception of the various potential infections it may be subject to, it must have a system in place that is flexible enough to deal with almost anything that comes along. Obviously, the systems are not perfect, but considering the wide range of pathogens, immune systems are remarkably efficient. In humans, there are two types of immune response to infection: the innate response, which is relatively nonspecific, and the adaptive, or acquired, response, which has more specificity.

The innate immune responses are common to all animals, and act on large classes of pathogens. For example, Toll-like receptors on phagocytes recognize a variety of bacterial surface molecules such as the flagellin specific to bacterial flagella, or the peptidoglycan components of bacterial cell walls. When these receptors are activated, the phagocyte goes into action, enveloping the offending bacteria or virus, and breaking

it down. This depends on recognizing the external surface, so bacteria or viruses that do not have a recognizable molecule on their surface are able to escape this particular line of defense.

Defensins, which are found on a variety of surfaces (skin, cornea, gut) as well as in circulation, are small (18-45 amino acids) cysteine-rich cationic proteins that bind to a variety of pathogenic viruses, bacteria, and fungi. It is unclear how they may work against viruses, other than perhaps attacking infected host cells, but against bacteria and fungi the mode of operation is generally to bind to the cell membrane and form a pore that allows ions and other small molecules to flow out killing the pathogen. Complement, a group of proteins (~20) circulating in the blood, can act similarly against pathogenic cells.

Finally, natural killer (NK) cells, lymphocytes that target any cell that does not carry cell surface proteins that are normally found on cells from the animal, can kill not only attacking cells, but virally infected cells that have stopped producing their normal proteins (including the recognition protein) because they are busy producing viral proteins. NK cells can even be effective against some cancer cells if they have downregulated cell surface protein expression as part of their de-differentiation and deadhesion.

The adaptive immune system, which is only found in vertebrates, is what most people think of when the human immune system is mentioned. We and other vertebrates also have an innate immune system, but all the molecules and cells that normally come to mind — antibodies, T-cells, B-cells — are part of the adaptive immune response. There are two components to the adaptive response, a humoral response, in which proteins (antibodies) floating in the blood bind to the infectious agent and prevent if from binding to cells or targeting it for the cellular response, which is mediated by T cells that can specifically recognize and kill the targeted pathogen.

Antibodies

Front and center in the adaptive immune response are antibodies. These proteins may be either secreted by or attached to the surface of B cells, the lymphocytes that differentiate either in bone marrow (adult) or liver (fetus), as opposed to those called T cells, which differentiate in the thymus gland. Incidentally, if you see sweetbreads on a menu, that would be thymus. Yum. You can take that seriously or sarcastically depending on how you think my tastes run.

Back to the antibodies. The different types of antibodies, IgA, IgD, IgE, IgG, and IgM, are all based on the IgG structure (fig. 18), which is roughly Y-shaped, and composed of two heavy chains and two light chains. These chains have disulfide bond-stabilized loops (recall the Ig-like loops in the cell adhesion molecules a few chapters back?), and the combination of the distal light chain loop and distal heavy chain loop make the antigen binding site. The antigen is defined as the molecule, or more specifically the part of a molecule that is recognized by the particular antibody. Since the antibody is meant to mediate highly specific recognition of a wide variety of invading pathogens, there must be a way to create at least as many different antibodies. This is made possible by the process of *DNA rearrangement*. This mechanism is also used to generate diversity in T-cell receptors, which are quite different structurally, but also need to be available with an extremely wide variety of specific binding sites.

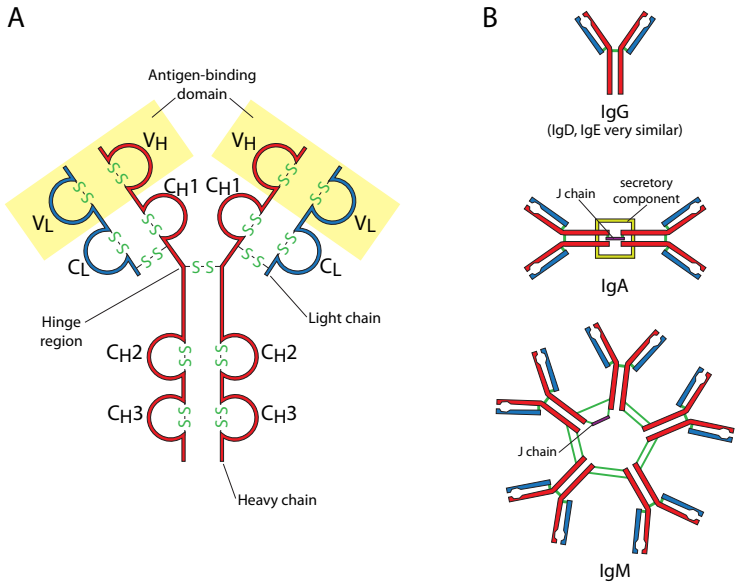


Figure 18. Antibodies. (A) an antibody is composed of two heavy chains (red) and two light chains (blue). Each has a variable region, and constant region(s). (B) IgA and IgM are built upon multiple IgG-like structures.

DNA Rearrangement

One of the central assumptions throughout our study of the cell has been that although the RNA and proteins in any cell may differ, any cell of a given organism other than the gametes should have the same DNA. This is not the case with B cells or T cells. In these cells, part of the maturation process is to create a unique arrangement of different domains to form a specific antibody (or T-cell receptor). The germline DNA, or the DNA that is found in all other somatic cells of the organism, contains many different such segments, but only a few are put together to make the antibody/TCR. This is a stochastic process, and with this kind of rearrangement happening on both heavy chain genes and two different light chain genes (designated κ and λ), there are well over 10 trillion (10^{13}) different combinations for generating immunoglobulins in humans, and even more combinations for T-cell receptors! How is this accomplished?

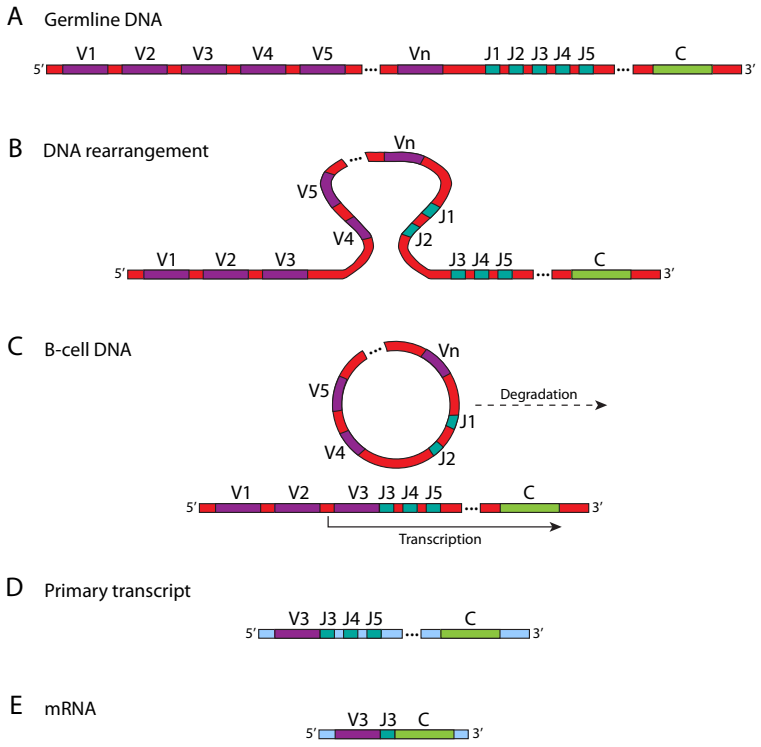


Figure 19. DNA rearrangement of a κ light chain gene. (A) The germline DNA has approximately 40 V region genes, 5 J segments, and one C region gene. (B) By action of the V(D)J recombinase, a random V region is brought close to a random J region, and the intervening sequence is cut out (C). (D) The gene may still contain multiple J segments, but RNA splicing removes all but one, leaving the final mRNA (E) with one V, one J, and one C region.

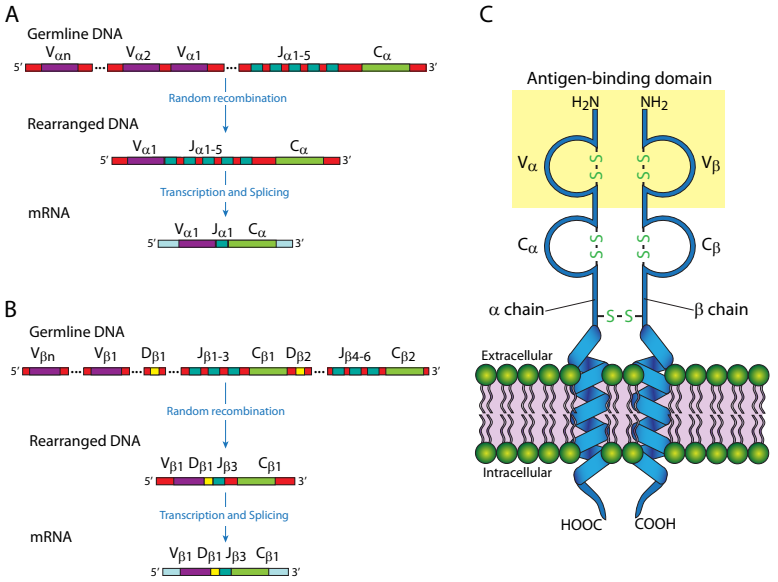


Figure 20. T-cell receptor genes also under DNA rearrangement to generate diversity like antibodies. In fact, there is actually greater diversity in TCR than in immunoglobulins. (A) rearrangement of α chain and (B) β chain. (C) protein structure of TCR.

Figure 19 shows the DNA rearrangements that take place in generating κ chain diversity. The λ chain locus has a slightly different arrangement, and has only 30 V genes, with 4 J segments and 4 C genes. The heavy chain has an extra domain: there are 40 V genes, which are linked to one of 25 D segments, then 6 potential J segments, and one C gene. These rearrangements, although they look something like the RNA splicing that we saw earlier in this course, are happening at the DNA level. Once it has happened, that cell and its progeny can no longer make the other combinations because those parts of the genome have been cut out and destroyed. This is distinctly different from alternative splicing of RNA, in which the genetic information is still there, and under different conditions could still generate other variations of the gene product.

The enzyme that produces this diversity is a complex called the V(D)J recombinase. The recombination occurs in two parts: first double-stranded breaks are made at recombination signal sequence (RSS) sites, then the breaks are repaired by the general double-stranded break repair mechanism. Depending on which J segment is chosen, there may be more than one left in the gene after the rearrangement. However, only the one closest to the V segment is used, and the others are spliced out of the primary transcript (by normal RNA splicing) in the process of connecting the C segment to the V and J for the final mRNA. Although this process generates great diversity, there is another mechanism that can generate further diversity under certain circumstances.

Somatic hypermutation causes rearranged V segments to mutate at 10^5 times the rate of other DNA! This mechanism is carried out by Activation-Induced Cytidine Deaminase (AID), which converts cytidines to uracils, generating a G:U mismatch that is "corrected" by repair polymerases without strong error-correction. This hypermutation is initiated by the activation of a B cell by recognizing and binding to a ligand. As we will see in the next paragraphs, when that happens, the B cell initiates rapid proliferation and this is when the somatic hypermutation takes effect, so that many of the B cells will carry highly similar but subtly different antibodies than the initial B cell that recognized and was activated by the antigen. The idea is some of these subtle mutation may lead to antibodies with higher affinity for the antigen and therefore faster response the next time this particular pathogen tries to infect the organism.

The reason that this kind of DNA rearrangement is necessary is that antibody "design" is not a reactive system, but a proactive system. A common misconception is that the immune system encounters a pathogen and creates antibodies that fit it. Unfortunately, there is no known mechanism by which a cell can "feel" the shape of something and create a protein that matches it. Instead, the immune system pre-emptively makes as many different antibodies (and TCRs) as possible, so that initially, most of the B and T cells in the body are actually genetically different. If an infection occurs, most of

RAG1 and RAG2, lymphocyte-specific recombination activating genes, recognize the RSS sites and make the double-stranded cuts. Once the cuts are made, the excised portion joins its ends together to form a circular signal joint (SJ) which is then degraded. The coding portions have asymmetric cut ends that fold into hairpin formations and prevent their fusion. These hairpins are broken by Artemis, a nuclease recruited by DNA-dependent protein kinase (DNA-PK), which also brings together XRCC4, XLF, DNA ligase IV, and a DNA polymerase. The XRCC and XLF alight the DNA ends, recruits a terminal transferase that randomly adds nucleotides to the ends, then DNA polymerase λ or μ fills in the overhangs, and the ligase completes the join. Interestingly, this process adds even more variability to the immunoglobulin, since Artemis cuts the hairpin at random, and the terminal transferase also adds nucleotides at random.

the B and T cells will bump into the pathogen and bounce right off, not recognizing it, but some of them will have the right antibody combination to bind to a part of the pathogenic invader. Although B cells can recognize surface antigens on their own, in most cases, a helper T cell is needed to activate the B cell (fig. 21). This process is initi-

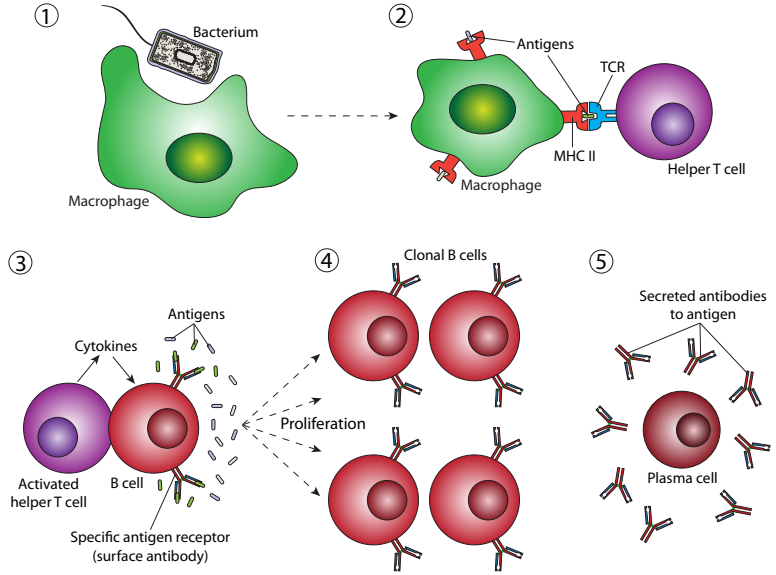


Figure 21. Activation of B cells by helper T-cells.

ated by a macrophage non-specifically ingesting a pathogen (1), breaking it apart, and presenting bits of it on its cell surface in partnership with MHC (major histocompatibility complex). A helper T-cell with a TCR that can recognize the antigen presented by the macrophage binds to it (2) and that leads to activation of the T cell. The activated helper T-cell binds to and activates a B cell (3) that has also bound to the antigen of interest, leading to massive B-cell proliferation (4), thus providing the body with many more copies of cells that have the right antibody to locate and fight the infection. This does two things: it provides lymphocyte reinforcements to specifically deal with a particular pathogen (but not other B cells, fig. 22), and once the pathogen has been eliminated, there is a larger circulating pool of these cells to respond more quickly to any subsequent infection by this particular type of pathogen. Finally, some of the B cells will differentiate into plasma cells (fig. 21-5), that secrete antibodies into the bloodstream to provide a humoral response. Others become memory cells, which are like B cells in that the antibody is on its cell surface and not secreted, but they can be thought of as "pre-activated" and can respond more quickly than naive B cells to re-infection.

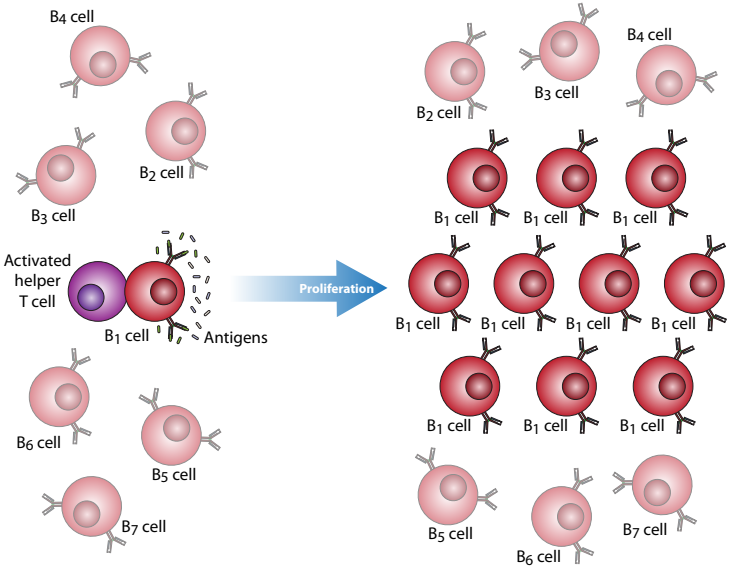


Figure 22. Amplification of only those B cells possessing antibodies that can recognize the infectious particle(s).

The big question that should have been lurking in the back of your mind through all this is, how do the antibodies and T-cell receptors tell what's foreign and what's part of one's own body? We'll get to that shortly. First, recall the activation of the helper T-cell. It occurs when the T-cell receptor recognizes an antigen from an ingested patho-

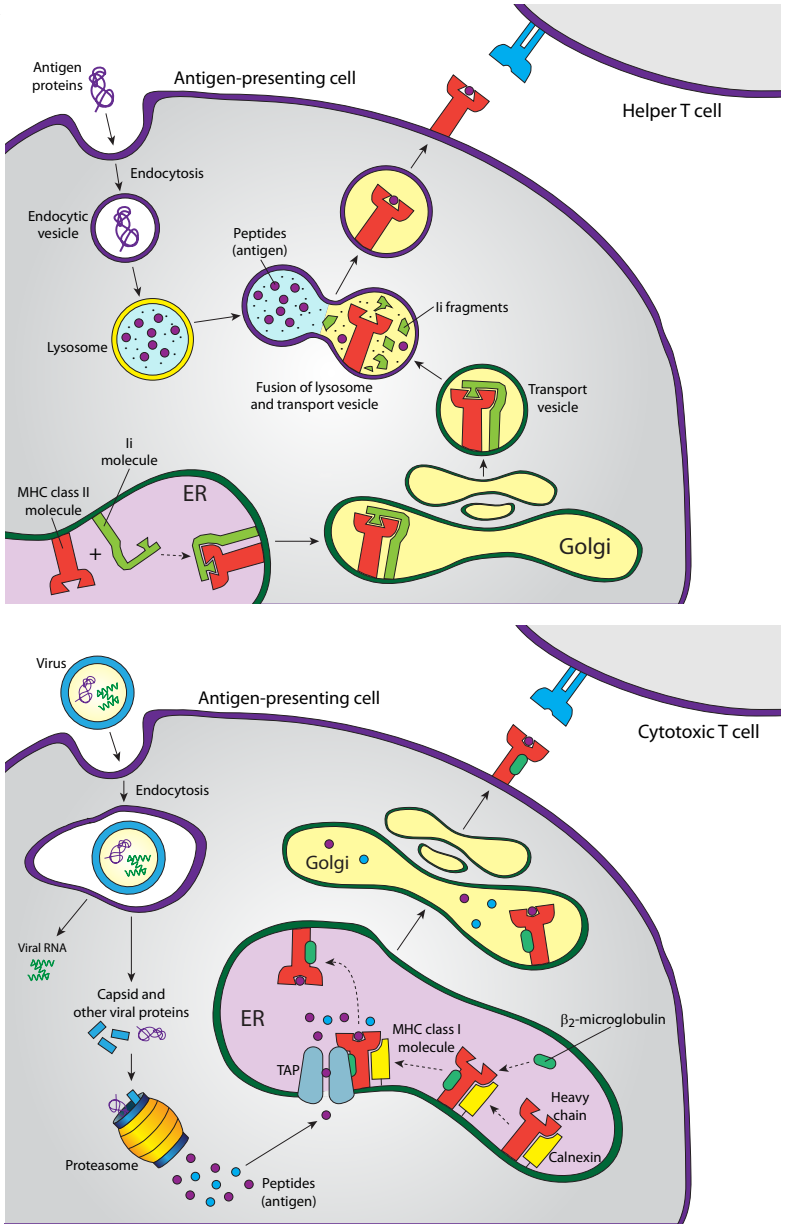


Figure 23. Antigen presentation by MHC class II (top) and class I (bottom) to T cells.

gen being presented by the MHC class II molecule on an antigen presenting cell (e.g. a macrophage). Figure 23 shows the pathway from ingestion of the pathogen to the presentation of its molecular parts on an MHC molecule. A similar pathway also applies to presentation of antigens on MHC class I molecules in conjunction with cytotoxic (killer) T cells. The cytotoxic T cells work against compromised cells, whether they are infected by a virus or another pathogen (fig. 24). There are two major pathways to

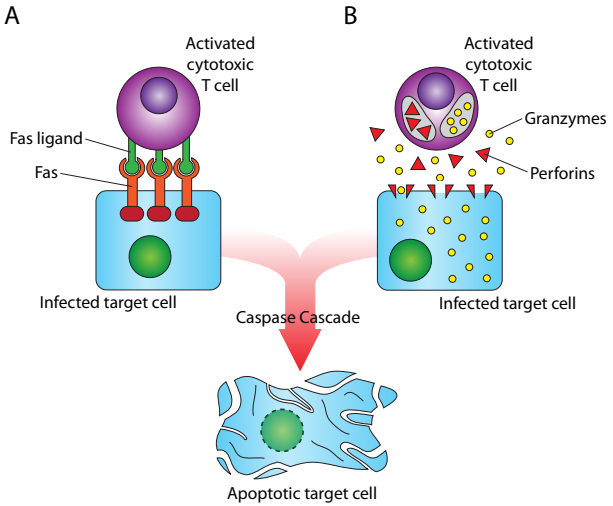


Figure 24. Cytotoxic T cells first recognize an infected cell by the T cell receptor, thich then leads to (A) binding of T-cell Fas ligand to Fas on the target cell, or alternatively, (B) secretion of granzymes and perforins. Both lead to activation of a caspase cascade and subsequent apoptosis of the infected cell.

killing the infected cell. One is the activation of a "death receptor", Fas, which induces a signal transduction cascade to activate caspases and apoptosis. The other pathway is the release of granzymes and perforins. The perforins drill into the membrane of the target cell and become pores that allow, among other things, granzymes to enter the cell, where they activate caspases by proteolysis, and again induce apoptosis. An important part of this is that the T cell receptor recognizes the antigen in combination with the MHC molecule that is presenting it. Furthermore, there are many variations of MHC molecules due there being 6 loci each with many known alleles.

So, what does any of this have to do with self vs non-self recognition? Early in the development of the immune system, the MHC does not present bits of digested pathogens, it presents bits of the organism's own cells that have gone through a proteasome or lysosome. At this early time, T cells behave somewhat differently, and if the T cell receptor binds strongly, the T cell commits apoptosis. This gets rid of TCR genes that strongly react to the organism's own cells. If the T cells do not react at all, apoptosis is also invoked, because the TCR genes that cannot recognize the MHC will not be useful in an immune response. Only those T cells that very weakly bind to the self-presenting MHC survive (fig. 25).

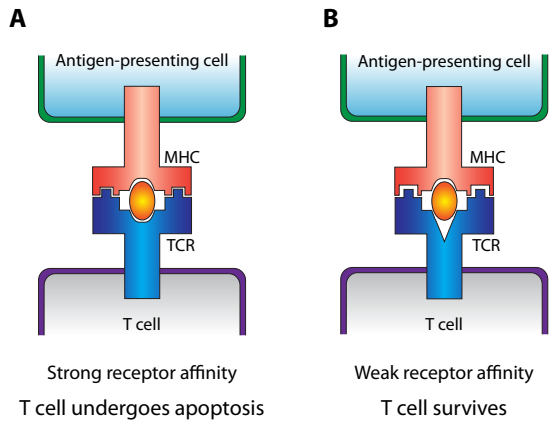


Figure 25. Self-recognition by T-cell receptor binding of MHC proteins presenting antigens derived from the organism's own cells. If there is strong recognition (A), the T cell dies to prevent cytotoxic attacks on its own cells. If it is weak and the TCR recognizes the MHC but not the antigen, the T cell survives (B).