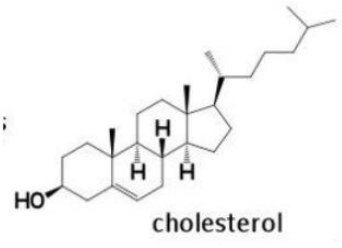
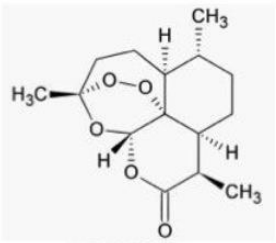
5.08 Exam #3 Solutions

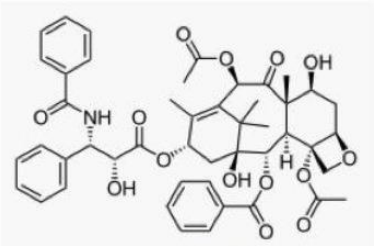
Name: _____



cholesterol

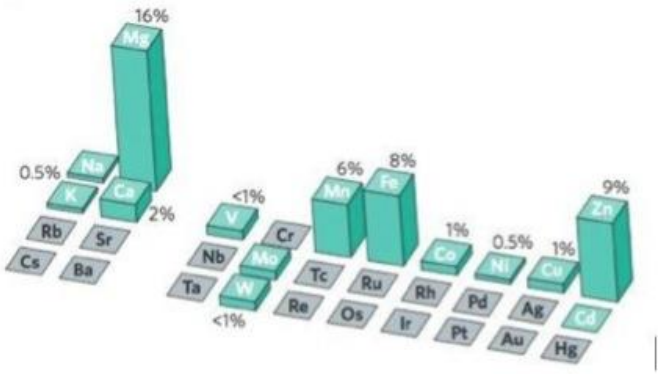


artemisinin



taxol

What do these natural products share in common?



Point Distribution:
Problem 1 (**20 points**)

1.

2.

3.

Problem 2 (**30 points**)

1.

2.

3.

4.

5.

6.

Problem 3 (**50 points**)
1.

2.

3.

4.

5.

6.

Total _____

1. **(20 points)** Farnesyl pyrophosphate (FPP) synthase has been extensively studied and catalyzes the reaction shown in Figure 1A. FPP synthase can also use 2-OPP as an alternative substrate (Figure 1B).

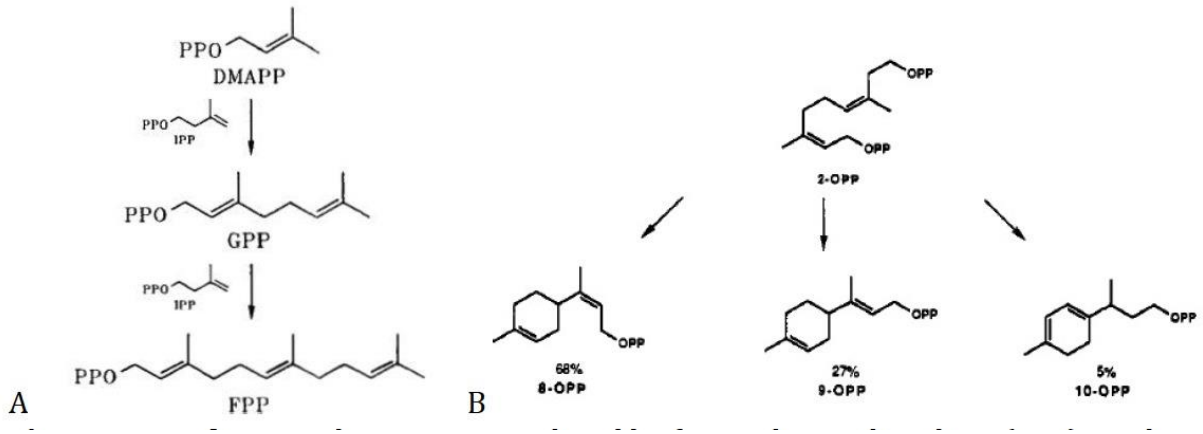
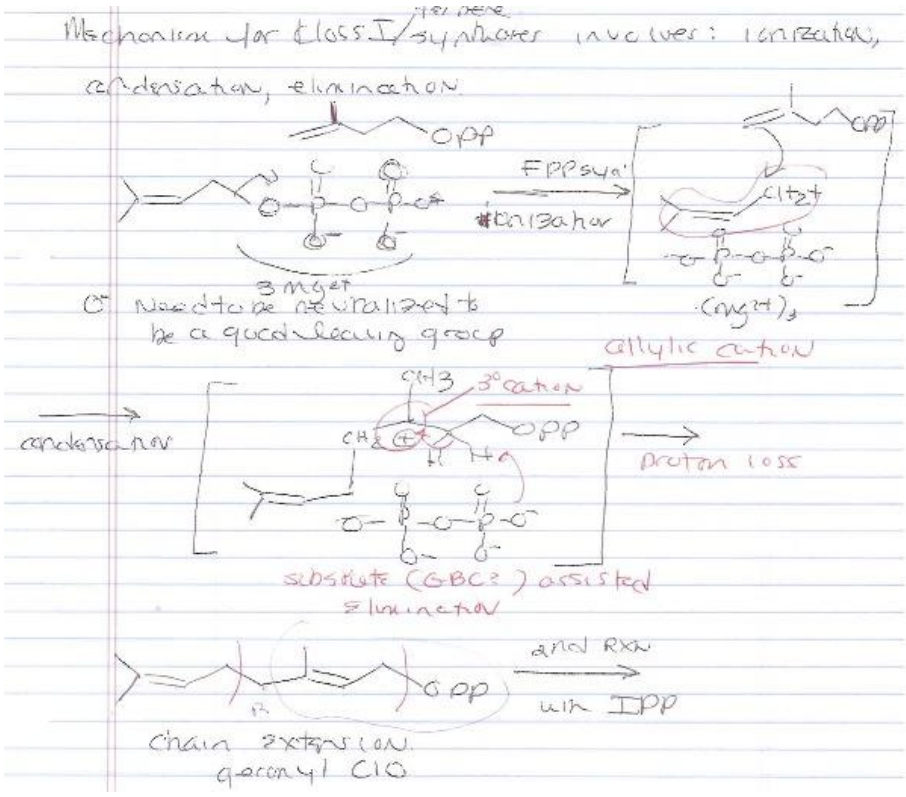


Figure 1. FPP synthase catalyzes the reaction in panel A of 2 IPP and 1 DMAPP to make FPP via the GPP intermediate and B. the reaction of FPP synthase with 2-OPP to form the three products shown.

Questions (20 points)

1. (**5 points**) Propose a detailed mechanism for formation of geranylPP from IPP and DMAPP. Draw out each step and show the proposed intermediate(s).

Three basic steps were discussed in class:ionization (loss of pyrophosphate) to from an allylic cation, carbon-carbon bond formation generating a second (tertiary) carbocation, followed by stereospecific loss of a proton. Many of you did not draw intermediates. These mechanisms are distinct from aldol and claisen reactions were carbanion intermediates are involve.

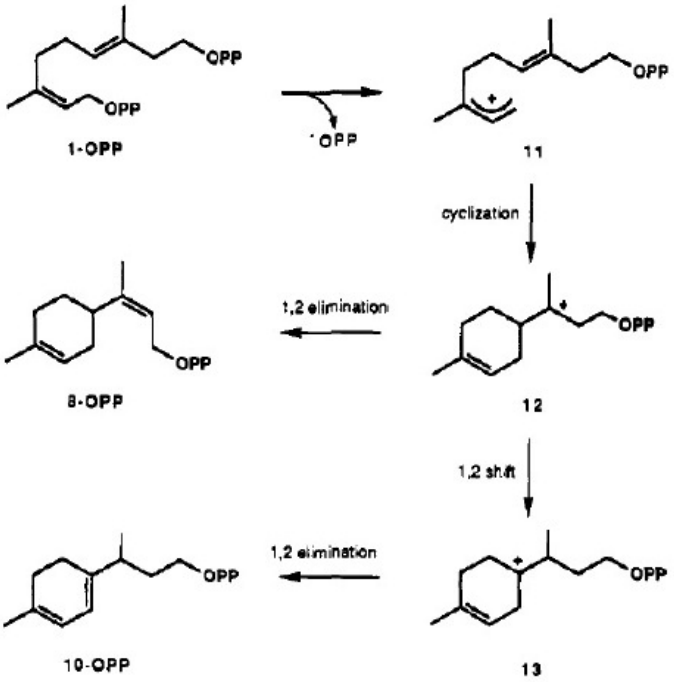


Mechanism taken from class notes.

2. (**10 points**) Another way to probe the presence of an intermediate you proposed in question 1 is to use substrate analogs. 2-OPP in Figure 1B was synthesized and studied with FPP sythase to gain mechanistic insight about the FPP synthase catalyzed reaction. The products of the reaction are also shown in Figure 1B.

   a. Propose a mechanism by which these products might be generated

This data was taken from JACS 115 1235 1993 a paper by the Poulter lab. The mechanism below was taken directly from the paper. Proton loss from 12 with or without rotation around the C-C bond could result in the 9-OPP or 8-OPP (trans and cis isomers). Enzymes in general are stereospecific and thus it would be unusual to have a general base in the active site that could abstract either proton. In fact, from class with the normal substrate, Mg-PPi is proposed to play that role. The observed products are consistent with what is known about the fate of carbocations: proton loss, hydride shifts, cyclizations). The authors tried to carry out rapid chemical quench studies and PPi exchange studies with ^{32}P labeled PPi to look for evidence for intermediates. All of those experiments failed so they turned to the use of substrate analogs. Carbocations are very unstable, so it is unlikely that PPi would dissociate from the active site to let the radiolabeled PPi bind. The intermediate would degrade. If you are having trouble understanding the general steps in these types o reactions please come see me.



   b. Given the types of reactions given in class that are prototypes for the chemistry you have described in question 1, what other product might be observed if you looked harder (had higher sensitivity methods of detection)?

Water can add to carbocations to give alcohols. Thus one might find an OH (alcohol) at any of the putative intermediate cations above. Two issues: 1. During the workup to look for other products, elimination of water could occur and 2. Water needs to get into the active site. Many of you had proton losses to generate alternative olefins. This is possible and the

amounts of products would be low because the enzyme is not set up to remove these alternative protons.

3. (**5 points**) The terpenome is composed of > 70,000 natural products. What is a unique feature of enzymes involved in making isoprenes and terpenes that distinguish these enzymes with most enzymatic reactions that you have learned about? (Hint, think about FPP)

FPP is a substrate for many terpene cyclases as described in class. An important part of product formation is the shape of the active site for FPP folding and perhaps the positioning of aromatics to stabilize π cation interactions of putative carbocation intermediates. These enzymes are in general much more promiscuous than other enzymes.

2. **(30 points)** This question was taken from J Lipid Res 49, 399 2008. HepG2 cells (liver cells) were grown in delipidated serum for up to 24 h and then the cells were transferred to fresh delipidated serum with 1 μM lovastatin; 50 μM sodium mevalonate (this concentration supplies the limited amount of this metabolite required for non-isoprenoid compounds essential for cell survival) in the presence or absence of sterols for the times indicated in Figure 2. The cell lysates were subjected to immunoblots with polyclonal antibodies against PCSK9 (proprotein convertase subtilisn/kexin type 9), low density lipoprotein receptor (LDLR), and sterol-regulatory element binding protein 2 (SREBP-2). The GAPDH protein was used as a loading control. P and C for PCSK9 are its proprotein (intracellular) and cleaved forms (extracellular).

A similar set of experiments in which subsequent to lovastatin induction, increasing concentrations of mevalonate were examined are shown in Figure 3.

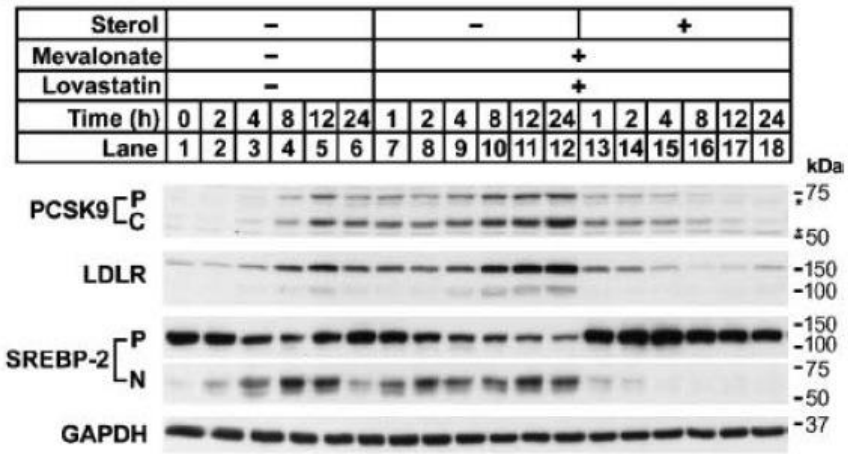


Figure 2. Time-course expression of PCSK9 in HepG2 cells after depletion or supplementation of sterols. HepG2 cells were grown and then switched at time 0 to delipidated serum for the indicated times with the indicated components (lanes 1-6); similar growth conditions in delipidated serum with 1 μM lovastatin and 50 μM doisum mevalonate in the absence (lanes 7-12) or the presence (lanes 13-18) of 1 μ□/mL cholesterol. After the indicated incubation times the cells were harvested, lysed and the whole cell lysates were subjected to immunoblot analysis with antibodies to PCSK9 or LDLR or SREBP-2. GAPDH was used as a control.

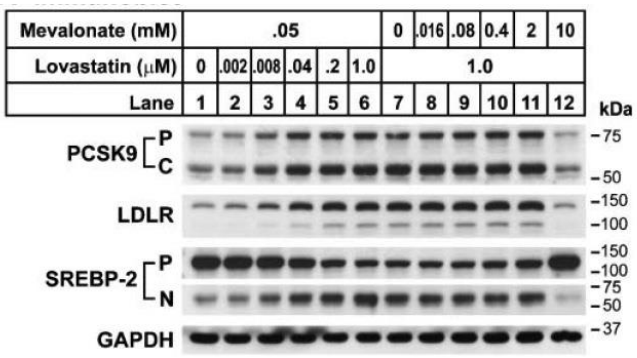
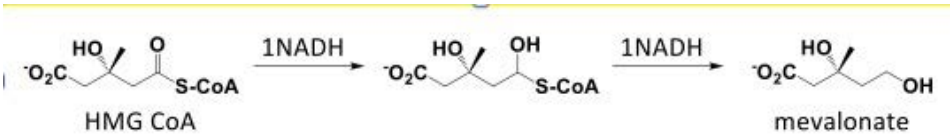


Figure 3 Induction of PCSK9 by lovastatin and the effects of different concentations of mevalonate. The growth conditions and analysis are similar to those described in Figure 1.

Questions (30 points)

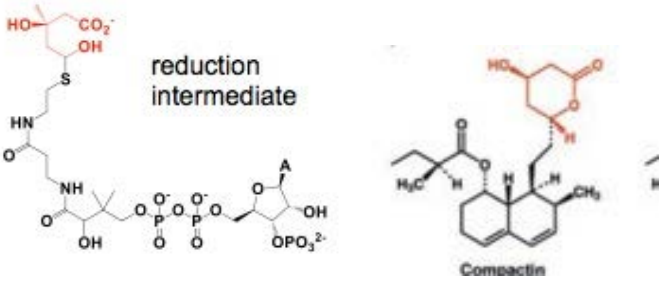
1. (**3 points**) In the cholesterol biosynthetic pathway, what is the rate-limiting step and what is the reaction catalyzed by this step (shown reactants and products)?

The rate limiting step is HMGCoA reductase: As noted in class the reductant is NADPH and not NADH

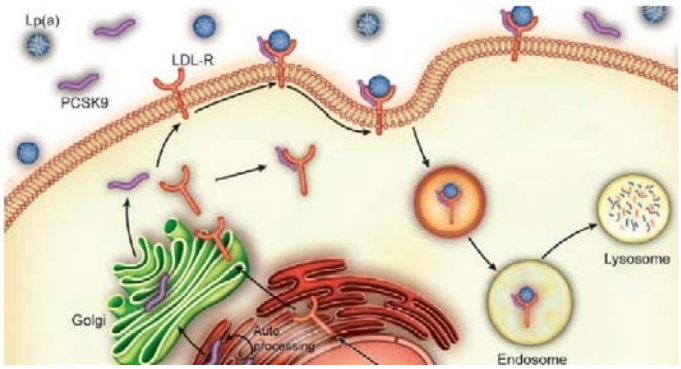


2. (**2 points**) What is the purpose of addition of lovastatin in the experiments described above and what are the expectations given its metabolic target?

Lovastatin is a competitive inhibitor of HMGCoA (they look alike) binding to HMGCoA reductase. Compactin is lovastatin and the lactone is the drug as it is taken more readily inot the cell. On ring opening of the lactone the red parts look alike. The black part is hydrophobic and enhances binding. It is distinct in all the statins.



3. (**5 points**) From recitation 8, provide the model for the role of PCSK9 in cholesterol homeostasis and why this protein is of interest clinically for lowering cholesterol levels.



Taken from the recitation handout. Hypothesis: The purple is the processed form of PCSK9 (pro (P) to C) that becomes extracellular. It binds to LDLR and causes it to edoscytose and go to the lysosome where the LDLR and PCSK9 are degraded. Thus the number of LDLRs are reduced and as a consequence the LDL levels in the plasma are raised.

4. (**4 points**) In Figure 2, describe what you see from the data.

The data in Figure 2 and 3 were taken from J of Lipid Res 49, 399409 (2008). Lanes 1-6 with no sterol, no MVA, and no lovastatin; up until 12 h, the levels of SREBP-2 in the nucleus increase, the LDLR levels increase (both to get more cholesterol) and the extracellular (C) PCSK9 levels also increase. They show parallel behavior.

In lanes 7-12, where there are no sterols, cholesterol biosynthesis is inhibited by lovastatin and MVA is at low levels, sufficient to keep cells alive; all three proteins nuclear SREBP, LDLR, and PCSK9 increase from 1 to 24 h. Again parallel behavior.

In lanes 13-18, where sterol is present, low levels of MVA are present and HMGCoA reductase is inhibited, all the SREBP remains in the ER as expected and the LDLR goes away both consistent with expectations when Ch present is present. The PCSK9 also goes away. The changes in the SREBP nuclear to ER movement, however, precedes the changes with the other proteins. This might suggest that SREBP controls transcriptionally the levels of LDLR (which we know is true), but also PCSK9 which we know nothing about.

5. (**6 points**) (In Figure 3, describe what you see from the data. Given the proposed function of PCSK9 described in recitation 8 and your answer to question 3, rationalize why the parallel observations for LDLR and PCSK9 in Figure 3 are a paradox.

Synthesis of all three proteins are induced by lovastatin inhibition of cholesterol biosynthesis. With SREBP, the amounts increase with higher levels of lovastatin to 1 μM. The levels of C form of PCSK9 increase, as do the levels of LDLR up to 1 μM. These studies reveal parallel trends, similar to those observed in Figure 2. The exception to the trends with all three proteins occurs at high concentrations of mevalonate (10 mM). Mevalonate is the product of HMGR which can be used in the biosynthetic pathway to overcome the lovastatin inhibition of HMGR. An important conclusion that one can draw from the data is that both LDLR and PCKS9 are dependent on the presence of sterols and perhaps regulated SREBP as a TF. The paradox is that as the amounts of PCKS9 protein in the extracellular form (C) increase, one would expect the LDLR and PCKS9 to be degraded in the lysosome (model in question 3). These studies do not

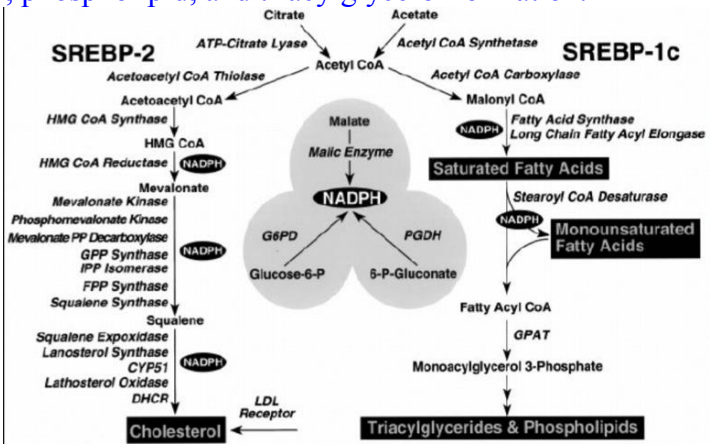
really address this model, but define regulation of PCKS9 at the transcriptional level. In fact we now know that PCSK9 has a putative SRE.

6. (**10 points**) In both Figures 2 and 3, the antibodies to SREBP-2 reveal two proteins labeled P and N.
    a. Why are there two apparent forms of SREBP-2? SREBP resides in the ER membrane when sterol levels are high.

When the levels of Ch are reduced (discussed in recitation 9) the SREBP is transported with SCAP to the golgi membranes where it is cleaved by two membrane located proteases (shown in part c). The soluble form is released to the cytosol and then is translocated to the nucleus where it functions as a transcription factor.

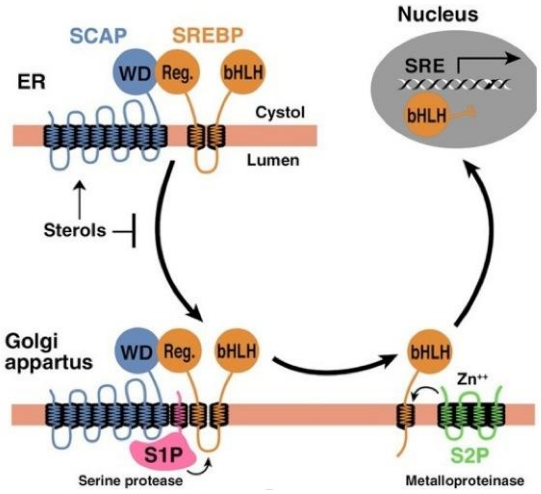
    b. What is the function of SREBP-2 (in a sentence describe the BIG picture)?

SREBP-2 is a transcription factor that regulates transcription of many of the proteins involved in Ch biosynthesis and homeostasis as well as many proteins involved in fatty acid metabolism, phospholipid, and triacylglycerol formation.



This is way more information than required. All the enzymes in italics are regulated by these TFs. This was the big picture given in class.

    c. Propose a cartoon model (like those described in class/notes) for the mechanism of active SREBP-2 production.



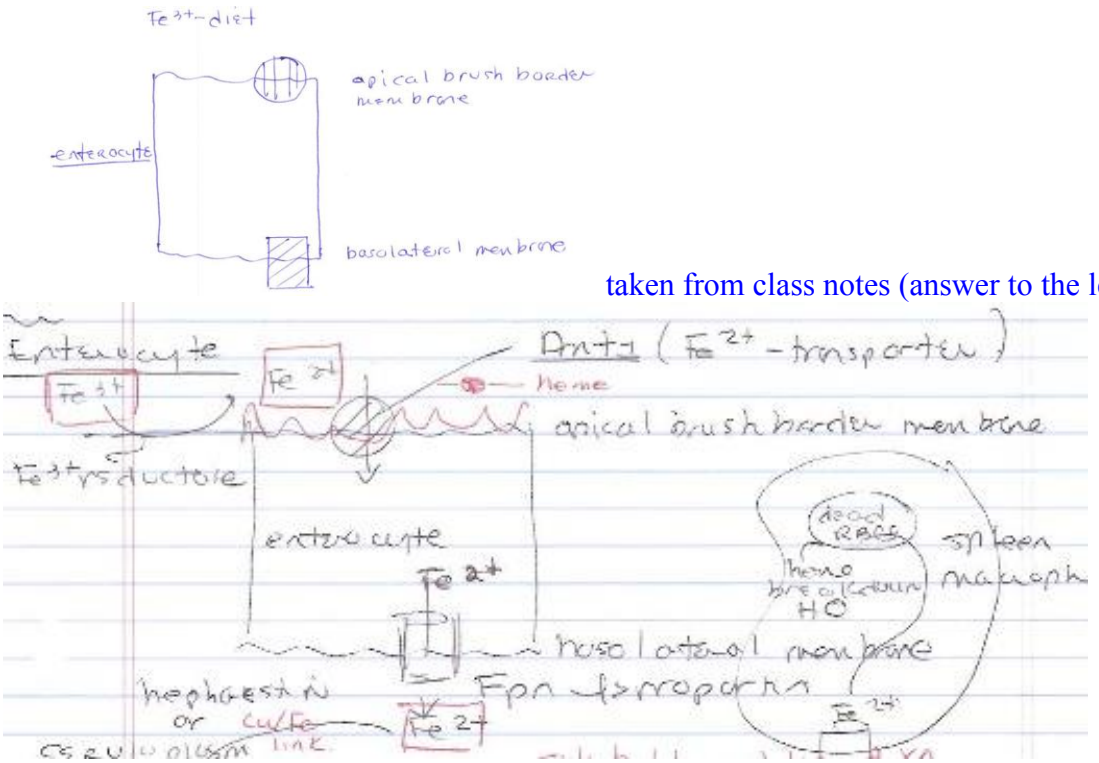
The answer did not need to be this detailed: ER localization, movement to golgi and cleavage in the membrane to release the cytosolic TR.

3. **(50 points**) Hepcidin is a peptide hormone that functions as the master regulator of systemic iron that maintains the levels of the plasma concentration of iron from 10 to 30 μM, despite fluctuations of iron in the diet. Studies predominantly on macrophages, involved in iron recycling from red blood cells, suggest that hepcidin functions by binding to Fpn extracellularly and targeting it for ubiquitination intracellularly which leads to Fpn degradation by the proteosome. The role of hepcidin has been postulated to be similar for iron uptake from the diet.

Very recently the function of hepcidin was examined in enterocytes (**Figure 4**) using cell culture (Caco cells) and in mouse slices from the duodenum (small intestine). Growth conditions similar to those used in the macrophage studies were chosen after much experimentation. The following experiments (**Figure 5, 6 and 7**) were carried out in cell culture with similar results in mouse duodenum slices. In the first set of experiments, optimized conditions (details not given), 0.2 μM hepcidin was incubated with Caco cells for 2 h and then western analysis was performed on crude membranes subsequent to SDS PAGE analysis and electroblotting, using antibodies specific for DMT1 and Fpn1. The results are shown in the western blot in **Figure 5A**, which was then quantitated as shown in **Figure 5B**.

The results shown in **Figure 5** suggested a second experiment in which Caco cells were incubated for one hour in the presence or absence of 50 μM MG-132 (a proteasome inhibitor) and then with or without hepcidin for 1 or 2 hours. Western analysis was performed on crude membranes as described above with antibodies specific for DMT1 and Fpn1. The results of the western analysis is shown in **Figure 6A** and the quantitative analysis is shown in **Figure 6B**.

The results in **Figure 6** suggested one last set of experiments shown in Figure 7. In this case the experiment is a little more complex in that the Caco cells were transfected with [IRE+]-DMT1-eGFP construct and studied under the similar conditions described in **Figures 5 and 6**. In this case the growth was carried out in the absence of presence of hepcidin over a 60 min time course. One additional experiment contained both hepcidin and PYR-41, an inhibitor of E1, the Ub activating enzyme. In this case the cells were lysed and immunoprecipitation carried out with GFP antibodies and then analyzed by western blotting using anti-DMT1 antibodies and antiubiquitin antibodies. The results are shown in Figure 7.



taken from class notes (answer to the left)

Figure 4. An enterocyte found in the small intestine. The circle and rectangle are proteins of interest in iron homeostasis. Note that diet iron enters enterocytes from the apical brush border membrane.

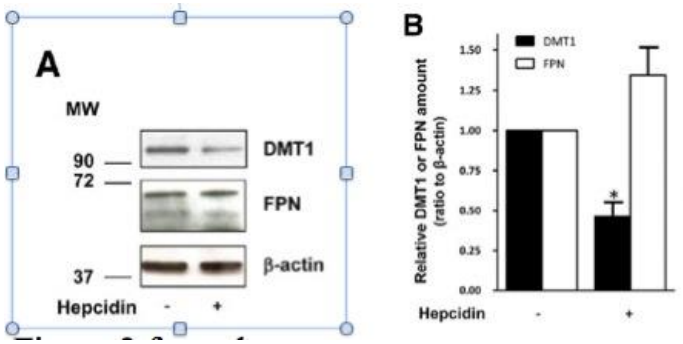


Figure 5. The effect of hepcidin on DMT1 and FPN protein in Caco cells. A. The cells were incubated for 2 hours with 0.2 μM hepcidin. At a defined time based on extensive experimentation (not described), crude membranes of the cells were analyzed by Western blotting using rabbit anti-mouse FPN antibody or with rabbit anti-rat DMT1 antibody. B. Quantitative analysis of the data in A.

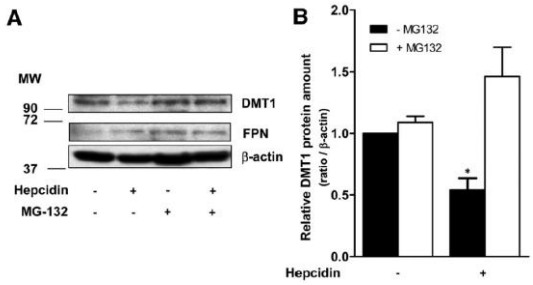


Figure 6. Western analysis of Caco cells incubated with in the presence or absence of hepcidin and the presence or absence of MG-132 a proteasome inhibitor. A the western blot using antibodies of DMT1 or FPN with actin as the loading standard. B. quantitative assessment of the results in A shown for DMT1. Note you were not given an "quantitative" analysis of the western data for Fpn. The eyeball method is challenging.

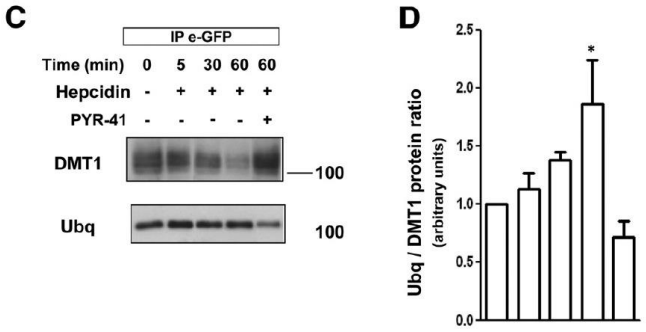


Figure 7. **C**. IP with E-GFP antibodies to GFP-DMT1 fusion protein under the conditions specified at the top of the Figure and over a 60 min period in the presence or absence of hepcidin. In one case PYR-41, an E1 activating enzyme inhibitor, was also present. The crude lysates were analyzed by western blotting with DMT1 antibodies or Ub antibodies. **D**. quantitation of part **C**.

Questions (**50 points**) :

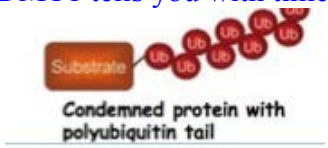
1. (**5 points**) In **Figure 4** you are given a cartoon of an enterocyte with a circular and rectangular protein. In addition you are given that Fe^{3+} is available from the diet. Write the name and function of the two proteins. What is missing in this diagram to allow Fe^{3+} to be taken into the enterocyte (assign proteins and draw the missing reaction on Figure 4)? See the picture above next to Figure 4
2. (**5 points**) Describe what you see in **Figure 5**. What is unusual about these observations relative to the proposed universal model by which hepcidin functions derived from similar experiments on macrophages?

This problem was taken from Gatroenterology 2011 140, 1261 to 1271. The levels of Fpn increase in the presence of hepcidin in the western in A which is quantified in B. This is the opposite result from the model from macrophage studies where hepcidin is given under the same environmental conditions. With DMT1, the FeII transporter, the DMT1 levels decrease in the westerns in A and quantitation by B in the presence of hepcidin. (Can obtain numbers from graph).

3. (**5 points**) Describe what you see in **Figure 6** and explain how these results provide a possible explanation for the results in **Figure 5**.

The addition of MG-132 (protease inhibitor) abolished the effect of hepcidin on DMT1 abundance, that is the DMT1 is no longer degraded as observed in Figure 5. With no hepcidin, no effect was observed (relative to Figure 5) in the presence or absence of MG-132. Moreover from the western analysis and "eyeball" the FPN protein levels appear to remain unchanged either with or without hepcidin. As noted above, no comment on this was expected

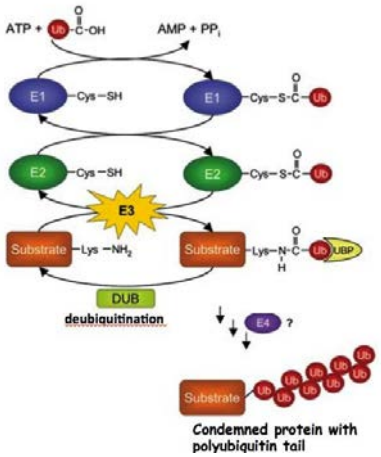
4. (**10 points**) Describe what you see in **Figure 7** in the first four lanes in **C** and **D**. Describe with a cartoon how these observations can further account for the observations in **Figures 5 and 6.**

Figure 7 is to determine whether proteosomal degradation of DMT1 suggested by the data in Figure 6 involves ubiquitination of the protein. The cells examined had DMT1 fused to GFP with an IRE to make sure it was expressed in a fashion similar to the endogenous gene construct. The immunoprecipitated DMT1 with the antibody to GFP (indicative of the amount of DMT1) and then the IP was analyzed with antibodies to Ubiquitin (and the ratio of Ub/DMT1) was quantitative.. As shown in C via westerns and quantified in D, hepcidin treatment of the cells induced rapid Ubiquitination (time t = 0) or there was endogenous Ubiquitinaiton, followed by DMT1 degradation with time over time 5 to 60 min shown by the loss of signal. In addition the ration of Ub/DMT1 tells you with time more Ub was present, indicative of targeting for degradation. 

One must have at least 4 Ubs for degradation targeting.

5. (**5 points**). In the fifth lane in **Figure 7** in the presence of PYR-41 a potent inhibitor of E1, explain the observed result in terms of cascade of events that appear to account for all the data in this figure.

The experiment in the last lane of Figure 7 showed the same 60 min time point as the preceding lane except that PYR-41 was present. ,Now the ubiquitinated DMT1 (similar to t = 0) was not degraded, but the Ub/DMT ratio decreased relative to the preceding three lanes. You were given that PYR-41 inhibits the E1 activating enzyme, an essential first step in the Ub process. Thus by inhibiting this step, the Ubiquitination was inhibited as indicated by the lower Ub/DMT1 ratio. The Ab to GFP-tagged DMT1 also shows that the protein is not degraded in the western analysis. You were not required to have the cartoon shown below.

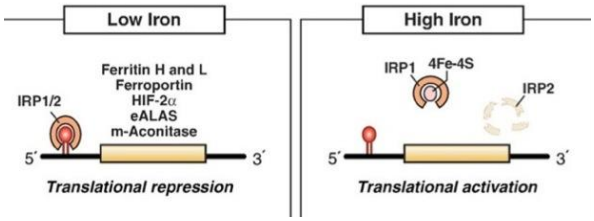


6. (**20 points**) Both DMT1 and Fpn play important role in homeostasis in are regulated at the translation level. Given the functions for these proteins draw the picture of the mRNA for each protein labeling the 5' and 3' ends. Show how you would expect each protein to be regulated at low iron and at high iron.

It is clear that hepcidin has tissue specific effects, in contrast to predictions in the literature based on studies with macrophages. The studies revealed that under the conditions, of physiological hepcidin, it rapidly reduced apical iron uptake by DMT1 in the enterocyte by

its degradation without altering the FPN content. The authors offer a number of explanations for differences in behavior in enterocytes vs macrophages. In the intestine, there may be a second form of FPN that is unable to interact with hepcidin. In fact a new FPN 1B transcript lacking the IRE sequence has been described in enterocytes and erythroid cells. You were not given this information. However no evidence for expression of this alternative protein form was provided in the paper. With DMT1 they propose that one has increased iron absorption due to stabilization of DMT1 by hepcidin in some fashion (directly? Indirectly?). The signaling pathway remains to be identified. At any rate, as indicated in PS9, our simplified models are a good place to start, but all need to be tested further especially when unpredicted/expected results are observed.

In answer to the question above. Ans taken from pp notes. At low iron DMT1 should be translated so that more FeII can be taken into the cell. Thus to get additional translation, the mRNA is stabilized by binding of apo IRP(1 or 2) to the 3' end of the mRNA. In the case of FPN, the model is that it exports FeII from the cell (see Figure 4) and at low iron one might want the iron to remain intracellular. Thus the translation of FPN is inhibited by binding of apo IRP(1 or 2) to the IRE at the 5' end of the mRNA.



Many of you were confused about the switch between high and low iron and the fact that only the apo IRPs bind to the iron responsive elements (IREs). If you are having trouble you should come back and talk. In a number of cases people were confused about the fact that this is regulation at the translational level and NOT the transcription level as we studied with SRE-BPs.

5.08J Biological Chemistry II

Spring 2016