

## **MCB6937: Methods to study prokaryotic transcriptional regulation (1 credit)**

### **Spring 2022**

MCB6937 will cover theoretical aspects as well as the methods available to identify and study prokaryotic proteins involved in transcriptional regulation. This course will explore specific methods used for the *in silico* and for the biochemical study of transcription factors.

### Introduction

The expression of genes encoded in the genome of all living organisms are switched on and off in response to external and internal stimuli. The dynamic interplay among the proteins that sense these changes and the modulation of gene expression is one of the most fascinating aspects studied by molecular biology. The knowledge gained from the study of the mechanisms involved in the modulation of the activity of these proteins helps in the understanding of the critical contribution of microorganisms to the host's health and disease status, their responses to environmental changes as well as their ability to use an unimaginable variety of chemicals as nutrients.

**Student Learning Outcomes** – After successful completion of this course, students will be able to:

- Understand the role of transcription factors in the regulation of gene expression.
- Compare and contrast one-, two- and three component transcription regulatory systems.
- Use *in silico* methods to identify, classify transcription factors and predict DNA and/or RNA binding sites in transcription factors.
- Select biochemical methods best suited to study different kind of transcription factors.
- Compare and contrast methods available for the biochemical study of chemicals and macromolecules as regulators of transcription factor's activity.
- Learn to set experimental designs directed to link molecular mechanisms to in vivo responses/consequences.

**Lectures:** Online through Canvas

**Instructor:** Dr. Graciela L Lorca

**Office:** Genetics Institute, Room 307

**WebPage:** Canvas (<https://ufl.instructure.com/>). Please select MCB6937.

**On line help with classroom technology:** <http://helpdesk.ufl.edu/>

**Pre-requisite:** MCB3020 or MCB3023

**Communication:** for questions regarding class and textbook content use the Discussion Board, for issues on Home Work Assignments, class organization check first the syllabus, the announcements and calendar on Canvas, then post your questions on the discussion board. For all other issues contact Dr. Graciela Lorca.

**VIRTUAL OFFICE HOURS: will be available every week (Tuesdays at 3PM) through the Zoom Conferences in Canvas.** To participate go to

Zoom Conferences in the left of your screen and join! You will receive a weekly reminder by email.

If you cannot make it to office hours you can request an appointment. Send an e-mail with three suggested times and I will choose one for us to meet.

**Contact Information: Use TEACHER in your emails through Canvas ONLY (personal emails should only be used in a case of emergency)**

Dr. Graciela L Lorca:

**Email (the most efficient):** ONLY use Canvas e-mail (If you do not have access to the e-learning platform and need to contact me for an **emergency**, use [glorca@ufl.edu](mailto:glorca@ufl.edu))

**Phone:** 273 8090 (please leave a message).

- **Discussion Board:** A discussion board is available in Canvas. It is very useful, please post and answer your questions on class content and organization there. Postings and answers are monitored by the instructor to make sure no mistakes get propagated. There are several discussion themes. Please post your questions in the adequate section.

**Material**

- **Textbook:** textbook is not required; this course is based on peer reviewed papers either available for free through the links provided or through the UF library (ejournals).

- **Suggested readings:** For each module, suggested readings will be posted as pdf documents on Canvas or as links to download them from PUBMED (see working list at the end of the document). Remember to connect to UF through VPN (if outside campus) before accessing the journals (<https://connect.ufl.edu/it/wiki/pages/glvpn.aspx>).

**Assessment of learning**

**Assignments (700 points):** Activities will be assigned by module. The activities include online research, use of online tools, virtual laboratory exercises and graded quizzes. The goal of these assignments is that the student keep-up with reading of the material on weekly basis. **The activities are mandatory and count towards the final grade. They should be completed by the deadline indicated on Canvas.**

**Critical integrative review (300 points):** The final paper will involve the search and writing of a critical review of at least 5 scientific articles on a **Bacterial transcription factor/family** (original research, no reviews will be allowed). The student will have to complete the review which contains the following five mandatory aspects: (1) *In vitro* experimental approaches to study TF, (2) *In vivo* experimental approaches to study TF, (3) Biochemical methods to study TF interactions with small molecules, (4) Biochemical methods to study TF interactions with proteins, and (5) Practical applications.

**LATE SUBMISSION POLICY:** a 5% deduction will be applied per day that the assignment/s is/are late.

*Make-Up policy:* Make-up assignments will ONLY be allowed with a VALID justification. If one assignment is missed, it will result in a score of 0 (see below for “Excused absences”).

*Excused absences:*

Documentation MUST be provided for absences caused by serious illness, accident, jury duty, or death in the immediate family. You must contact the instructor **as soon as possible** of the missed assignment and I will arrange an alternative time for the submission.

Requirements for class attendance and make-up assignments and other work are consistent with university policies that can be found at: <https://catalog.ufl.edu/ugrad/current/regulations/info/attendance.aspx>.

**Grades and Grade Points**

For information on current UF policies for assigning grade points, see <https://catalog.ufl.edu/UGRD/academic-regulations/grades-grading-policies/>

**Grading:** Straight scale

**Grading Scale**

<b>A</b>	900 or above
<b>A-</b>	860-899
<b>B+</b>	830-859
<b>B</b>	790-829
<b>B-</b>	750-789
<b>C+</b>	720-749
<b>C</b>	690-719
<b>C-</b>	660-689
<b>D+</b>	630-659
<b>D</b>	600-629
<b>D-</b>	570-599
<b>E</b>	560 or below

# Schedule of Classes

Week	Unit	Module. Topic
W1	Unit 1	<b>Transcription factors (TF): definitions, history and classification</b> 1. Review on the prokaryotic transcriptional machinery and its components Review on prokaryotic mechanisms of regulation of gene expression 2. Classification of TF into families: One-, two and three component systems 3. Genomic occurrence of TF encoding genes and their roles in environmental adaptations
W2	Unit 2	<b><i>In silico, in vitro</i> and <i>in vivo</i> approaches to study TF</b> 4. Genomic identification of TF and their binding sites: use of online tools 5. <i>In vivo</i> Experimental approaches to study TF 6. <i>In vitro</i> Experimental approaches to study TF
W3		7. Biochemical methods to study TF interactions with small molecules 8. Biochemical methods to study TF interactions with proteins
W4	Unit 3	<b>Practical applications: Manipulation of transcriptional regulation</b> 9. Applications in synthetic biology and as targets of new antimicrobials

## University of Florida Policies

### **Grades and Grade Points**

For information on current UF policies for assigning grade points, see <https://catalog.ufl.edu/UGRD/academic-regulations/grades-grading-policies/>

### **Attendance and Make-Up Work**

Requirements for class attendance and make-up exams, assignments and other work are consistent with university policies that can be found at: <https://catalog.ufl.edu/UGRD/academic-regulations/attendance-policies/>

### **Services for Students with Disabilities**

Students with disabilities who experience learning barriers and would like to request academic accommodations should connect with the disability Resource Center (<https://disability.ufl.edu/get-started/>). It is important for students to share their accommodation letter with their instructor and discuss their access needs, as early as possible in the semester.

### **Campus Helping Resources**

#### Health and Wellness

- U Matter, We Care: If you or someone you know is in distress, please contact [umatter@ufl.edu](mailto:umatter@ufl.edu), 352-392-1575, or visit U Matter, We Care website (<https://umatter.ufl.edu/>) to refer or report a concern and a team member will reach out to the student in distress.

- Counseling and Wellness Center: Visit the Counseling and Wellness Center website (<https://counseling.ufl.edu/>) or call 352-392-1575 for information on crisis services as well as non-crisis services.
- Student Health Care Center: Call 352-392-1161 for 24/7 information to help you find the care you need, or visit the Student Health Care Center website (<https://shcc.ufl.edu/>).
- University Police Department: Visit UF Police Department website (<https://police.ufl.edu/>) or call 352-392-1111 (or 9-1-1 for emergencies).  
 UF Health Shands Emergency Room / Trauma Center: For immediate medical care call 352-733-0111 or go to the emergency room at 1515 SW Archer Road, Gainesville, FL 32608; Visit the UF Health Emergency Room and Trauma Center website (<https://ufhealth.org/emergency-room-trauma-center>).
- GatorWell Health Promotion Services: For prevention services focused on optimal wellbeing, including Wellness Coaching for Academic Success, visit the GatorWell website (<https://gatorwell.ufsa.ufl.edu/>) or call 352-273-4450.

### **Academic Resources**

- E-learning technical support: Contact the UF Computing Help Desk at 352-392-4357 or via e-mail at [helpdesk@ufl.edu](mailto:helpdesk@ufl.edu).
- Career Connections Center: Reitz Union Suite 1300, 352-392-1601. Career assistance and counseling services (<https://career.ufl.edu/>).
- Library Support: Various ways to receive assistance with respect to using the libraries or finding resources (<https://uflib.ufl.edu/>).
- Teaching Center: Broward Hall, 352-392-2010 or to make an appointment 352-392-6420. General study skills and tutoring (<https://teachingcenter.ufl.edu/>).
- Writing Studio: 2215 Turlington Hall, 352-846-1138. Help brainstorming, formatting, and writing papers (<https://writing.ufl.edu/writing-studio/>).
- Student Complaints On-Campus: Visit the Student Honor Code and Student Conduct Code webpage for more information (<https://sccr.dso.ufl.edu/policies/student-honor-%20code-student-conduct-code/>).
- On-Line Students Complaints: View the Distance Learning Student Complaint Process (<https://distance.ufl.edu/state-authorization-status/#student-complaint>).

### **Course Evaluation**

Students are expected to provide professional and respectful feedback on the quality of instruction in this course by completing course evaluations online via GatorEvals. Guidance on how to give feedback in a professional and respectful manner is available at <https://gatorevals.aa.ufl.edu/students/>. Students will be notified when the evaluation period opens, and can complete evaluations through the email they receive from GatorEvals, in their Canvas course menu under GatorEvals, or via <https://ufl.bluera.com/ufl/>. Summaries of course evaluation results are available to students at <https://gatorevals.aa.ufl.edu/public-results/>.

### **Class demeanor**

Opinions held by other students should be respected in discussion, and conversations that do not

contribute to the discussion should be held at minimum, if at all.

### **Netiquette guide for online courses**

It is important to recognize that the online classroom is in fact a classroom, and certain behaviors are expected when you communicate with both your peers and your instructors. These guidelines for online behavior and interaction are known as netiquette.

<http://teach.ufl.edu/wp-content/uploads/2012/08/NetiquetteGuideforOnlineCourses.pdf>

### **Academic Honesty**

As a student at the University of Florida, you have committed yourself to uphold the Honor Code, which includes the following pledge: *"We, the members of the University of Florida community, pledge to hold ourselves and our peers to the highest standards of honesty and integrity."* You are expected to exhibit behavior consistent with this commitment to the UF academic community, and on all work submitted for credit at the University of Florida, the following pledge is either required or implied: *"On my honor, I have neither given nor received unauthorized aid in doing this assignment."*

It is assumed that you will complete all work independently in each course unless the instructor provides explicit permission for you to collaborate on course tasks (e.g. assignments, papers, quizzes, exams). Furthermore, as part of your obligation to uphold the Honor Code, you should report any condition that facilitates academic misconduct to appropriate personnel. It is your individual responsibility to know and comply with all university policies and procedures regarding academic integrity and the Student Honor Code. Violations of the Honor Code at the University of Florida will not be tolerated. Violations will be reported to the Dean of Students Office for consideration of disciplinary action. For more information regarding the Student Honor Code, please see: <http://www.dso.ufl.edu/sccr/process/student-conduct-honor-code>.

### **Additional comments regarding academic integrity:**

Students are encouraged to discuss material with each other from the course, help each other understand concepts, study together, and even discuss assessment questions with each other once the quiz window is closed. However, the following is considered academic dishonesty, and I expect that no student will ever do any of the following:

- Have another person complete a quiz in this course
- Copy another student's quiz in this course
- Collaborate with anyone during a quiz in this course
- Discuss the questions and answers of a quiz with other students while the quiz window is still open
- Manipulate and/or distribute any materials provided in this course for any purpose (including course lecture slides).
- Use any materials provided by a previous student in the course

### **Software Use**

All faculty, staff and students of the university are required and expected to obey the laws and legal agreements governing software use. Failure to do so can lead to monetary damages and/or criminal penalties for the individual violator. Because such violations are also against university policies and rules, disciplinary action will be taken as appropriate.

### **Microsoft Office 365 Software is free for UF students**

<http://www.it.ufl.edu/gatorcloud/free-office-365-downloads/>

**Other free software is available at:**

<http://www.software.ufl.edu/>

To check for availability of the media and technical requirements, contact the UF Computing Help Desk at (352)392-HELP(4357).

**University of Florida Complaints Policy and Student Complaint Process**

**Most problems, questions and concerns about the course will be resolved by professionally communicating with the instructors.**

The University of Florida believes strongly in the ability of students to express concerns regarding their experiences at the University. The University encourages its students who wish to file a written complaint to submit that complaint directly to the department that manages that policy.

If a problem really cannot be resolved by communicating with the instructor or the TAs you can contact

- Residential Course: <https://sccr.dso.ufl.edu/policies/student-honor-code-student-conduct-code/>.
- Online Course: <http://www.distance.ufl.edu/student-complaint-process>.

This said, professionalism is a two-way-street. Unprofessional behavior of students includes, among other things: lack of communication, blaming other people or external factors, lying, affecting others negatively in a group or in the class, not accepting criticism and not being proactive in solving problems or seeking help. Furthermore, faculty often have family and other obligations to tend to. Over the weekend, replies to your inquiries or questions maybe delayed. If a student is lacking professionalism repeatedly, the instructor has the rights to file formal complaint against the student through the Dean of Student office.

## **Suggested Readings and Sources**

### **Module 1**

- Any General Microbiology Book: Chapters on bacterial transcription and gene regulation. In example:
- Diversity, versatility and complexity of bacterial gene regulation mechanisms: opportunities and drawbacks for applications in synthetic biology. Bervoets I, Charlier D. FEMS Microbiol Rev. 2019 May 1;43(3):304-339. doi: 10.1093/femsre/fuz001. PMID: 30721976

### **Module 2**

- Transcription factor-based biosensors enlightened by the analyte. Fernandez-López R, Ruiz R, de la Cruz F, Moncalián G. Front Microbiol. 2015 Jul 1;6:648. doi: 10.3389/fmicb.2015.00648. eCollection 2015. PMID: 26191047

- Allostery in the LacI/GalR family: variations on a theme. Swint-Kruse L, Matthews KS. *Curr Opin Microbiol.* 2009 Apr;12(2):129-37. doi: 10.1016/j.mib.2009.01.009. Epub 2009 Mar 5. PMID: 19269243
- Phylogeny of the bacterial superfamily of Crp-Fnr transcription regulators: exploiting the metabolic spectrum by controlling alternative gene programs. Körner H, Sofia HJ, Zumft WG. *FEMS Microbiol Rev.* 2003 Dec;27(5):559-92. doi: 10.1016/S0168-6445(03)00066-4. PMID: 14638413
- Bacterial sensor kinases: diversity in the recognition of environmental signals. Krell T, Lacal J, Busch A, Silva-Jiménez H, Guazzaroni ME, Ramos JL. *Annu Rev Microbiol.* 2010;64:539-59. doi: 10.1146/annurev.micro.112408.134054. PMID: 20825354
- The three-component signalling system HbpS-SenS-SenR as an example of a redox sensing pathway in bacteria. Ortiz de Orué Lucana D, Groves MR. *Amino Acids.* 2009 Sep;37(3):479-86. doi: 10.1007/s00726-009-0260-9. Epub 2009 Mar 4. PMID: 19259771
- On prokaryotic intelligence: strategies for sensing the environment. Marijuán PC, Navarro J, del Moral R. *Biosystems.* 2010 Feb;99(2):94-103. doi: 10.1016/j.biosystems.2009.09.004. Epub 2009 Sep 23. PMID: 19781596

### **Module 3**

- The repertoire of DNA-binding transcription factors in prokaryotes: functional and evolutionary lessons. Perez-Rueda E, Martínez-Núñez MA. *Sci Prog.* 2012;95(Pt 3):315-29. doi: 10.3184/003685012X13420097673409. PMID: 23094327
- Dissecting the protein architecture of DNA-binding transcription factors in bacteria and archaea. Rivera-Gómez N, Martínez-Núñez MA, Pastor N, Rodríguez-Vazquez K, Perez-Rueda E. *Microbiology (Reading).* 2017 Aug;163(8):1167-1178. doi: 10.1099/mic.0.000504. Epub 2017 Aug 17. PMID: 28777072
- Abundance, diversity and domain architecture variability in prokaryotic DNA-binding transcription factors. Perez-Rueda E, Hernandez-Guerrero R, Martínez-Núñez MA, Armenta-Medina D, Sanchez I, Ibarra JA. *PLoS One.* 2018 Apr 3;13(4):e0195332. doi: 10.1371/journal.pone.0195332. eCollection 2018. PMID: 29614096

### **Module 4**

- RegPrecise 3.0--a resource for genome-scale exploration of transcriptional regulation in bacteria. Novichkov PS, Kazakov AE, Ravcheev DA, Leyn SA, Kovaleva GY, Sutormin RA, Kazanov MD, Riehl W, Arkin AP, Dubchak I, Rodionov DA. *BMC Genomics.* 2013 Nov 1;14:745. doi: 10.1186/1471-2164-14-745. PMID: 24175918
- P2CS: updates of the prokaryotic two-component systems database Philippe Ortet, David E. Whitworth, Catherine Santaella, Wafa Achouak, Mohamed Barakat *Nucleic Acids Res.* 2015 Jan 28; 43(Database issue): D536–D541. Published online 2014 Oct 16. doi: 10.1093/nar/gku968 PMCID: PMC4384028



- P2RP: a web-based framework for the identification and analysis of regulatory proteins in prokaryotic genomes Mohamed Barakat, Philippe Ortet, David E Whitworth BMC Genomics. 2013; 14: 269. Published online 2013 Apr 20. doi: 10.1186/1471-2164-14-269 PMID: PMC3637814

## **Module 5**

- Tools to map target genes of bacterial two-component system response regulators. Rajeev L, Garber ME, Mukhopadhyay A. Environ Microbiol Rep. 2020 Jun;12(3):267-276. doi: 10.1111/1758-2229.12838. Epub 2020 Apr 5. PMID: 32212247
- Functional Transcriptomics for Bacterial Gene Detectives. Perez-Sepulveda BM, Hinton JCD. Microbiol Spectr. 2018 Sep;6(5). doi: 10.1128/microbiolspec.RWR-0033-2018. PMID: 30215343
- A survey of best practices for RNA-seq data analysis. Conesa A, Madrigal P, Tarazona S, Gomez-Cabrero D, Cervera A, McPherson A, Szczesniak MW, Gaffney DJ, Elo LL, Zhang X, Mortazavi A. Genome Biol. 2016 Jan 26;17:13. doi: 10.1186/s13059-016-0881-8. PMID: 26813401
- Erratum to: A survey of best practices for RNA-seq data analysis. Conesa A, Madrigal P, Tarazona S, Gomez-Cabrero D, Cervera A, McPherson A, Szczesniak MW, Gaffney DJ, Elo LL, Zhang X, Mortazavi A. Genome Biol. 2016 Aug 26;17(1):181. doi: 10.1186/s13059-016-1047-4. PMID: 27565134.
- Rapid engineering of bacterial reporter gene fusions by using Red recombination. Gerlach RG, Hölzer SU, Jäckel D, Hensel M. Appl Environ Microbiol. 2007 Jul;73(13):4234-42. doi: 10.1128/AEM.00509-07. Epub 2007 May 18. PMID: 17513596

## **Module 6**

- Tools to map target genes of bacterial two-component system response regulators. Rajeev L, Garber ME, Mukhopadhyay A. Environ Microbiol Rep. 2020 Jun;12(3):267-276. doi: 10.1111/1758-2229.12838. Epub 2020 Apr 5. PMID: 32212247
- Systematic characterization of protein-DNA interactions. Xie Z, Hu S, Qian J, Blackshaw S, Zhu H. Cell Mol Life Sci. 2011 May;68(10):1657-68. doi: 10.1007/s00018-010-0617-y. Epub 2011 Jan 5. PMID: 21207099
- Identifying and characterizing Hfq-RNA interactions. Faner MA, Feig AL. Methods. 2013 Sep 15;63(2):144-59. doi: 10.1016/j.ymeth.2013.04.023. Epub 2013 May 23. PMID: 23707622

## **Module 7**

- High-Throughput Screening to Identify Chemoreceptor Ligands. Fernández M, Ortega Á, Rico-Jiménez M, Martín-Mora D, Daddaoua A, Matilla MA, Krell T. Methods Mol Biol. 2018;1729:291-301. doi: 10.1007/978-1-4939-7577-8\_23. PMID: 29429099

- Insights into Protein-Ligand Interactions: Mechanisms, Models, and Methods. Du X, Li Y, Xia YL, Ai SM, Liang J, Sang P, Ji XL, Liu SQ. *Int J Mol Sci*. 2016 Jan 26;17(2):144. doi: 10.3390/ijms17020144. PMID: 26821017
- Surface plasmon resonance spectroscopy for characterisation of membrane protein-ligand interactions and its potential for drug discovery. Patching SG. *Biochim Biophys Acta*. 2014 Jan;1838(1 Pt A):43-55. doi: 10.1016/j.bbamem.2013.04.028. Epub 2013 May 9. PMID: 23665295

### **Module 8**

- The study of protein-protein interactions in bacteria. Velasco-García R, Vargas-Martínez R. *Can J Microbiol*. 2012 Nov;58(11):1241-57. doi: 10.1139/w2012-104. Epub 2012 Oct 29. PMID: 23145822 <https://cdnsiencepub.com/doi/pdf/10.1139/w2012-104>
- A beta-galactosidase-based bacterial two-hybrid system to assess protein-protein interactions in the correct cellular environment. Borloo J, De Smet L, Vergauwen B, Van Beeumen JJ, Devreese B. *J Proteome Res*. 2007 Jul;6(7):2587-95. doi: 10.1021/pr070037j. Epub 2007 Jun 1. PMID: 17539672
- Yeast Two-Hybrid Assay to Identify Interacting Proteins. Paiano A, Margiotta A, De Luca M, Bucci C. *Curr Protoc Protein Sci*. 2019 Feb;95(1):e70. doi: 10.1002/cpps.70. Epub 2018 Aug 21. PMID: 30133175
- A Comparison of Two-Hybrid Approaches for Detecting Protein-Protein Interactions. Mehla J, Caufield JH, Sakhawalkar N, Uetz P. *Methods Enzymol*. 2017;586:333-358. doi: 10.1016/bs.mie.2016.10.020. Epub 2017 Jan 5. PMID: 28137570

### **Module 9**

- Diversity, versatility and complexity of bacterial gene regulation mechanisms: opportunities and drawbacks for applications in synthetic biology. Bervoets I, Charlier D. *FEMS Microbiol Rev*. 2019 May 1;43(3):304-339. doi: 10.1093/femsre/fuz001. PMID: 30721976
- Two-Component Signal Transduction Systems of Pathogenic Bacteria As Targets for Antimicrobial Therapy: An Overview. Tiwari S, Jamal SB, Hassan SS, Carvalho PVSD, Almeida S, Barh D, Ghosh P, Silva A, Castro TLP, Azevedo V. *Front Microbiol*. 2017 Oct 10;8:1878. doi: 10.3389/fmicb.2017.01878. eCollection 2017. PMID: 29067003
- Transcription factor-based biosensors enlightened by the analyte. Fernandez-López R, Ruiz R, de la Cruz F, Moncalián G. *Front Microbiol*. 2015 Jul 1;6:648. doi: 10.3389/fmicb.2015.00648. eCollection 2015. PMID: 26191047
- Pharmacological manipulation of transcription factor protein-protein interactions: opportunities and obstacles. Fontaine F, Overman J, François M. *Cell Regen*. 2015 Mar 12;4(1):2. doi: 10.1186/s13619-015-0015-x. eCollection 2015. PMID: 25848531
- Drug Repurposing: Tolfenamic Acid Inactivates PrbP, a Transcriptional Accessory Protein in *Liberibacter asiaticus*. Gardner CL, Pagliai FA, Pan L, Bojilova L, Torino MI,

Lorca GL, Gonzalez CF. *Front Microbiol.* 2016 Oct 18;7:1630. doi: 10.3389/fmicb.2016.01630. eCollection 2016. PMID: 27803694

- The transcriptional activator LdtR from 'Candidatus Liberibacter asiaticus' mediates osmotic stress tolerance. Pagliai FA, Gardner CL, Bojilova L, Sarnegrim A, Tamayo C, Potts AH, Teplitski M, Folimonova SY, Gonzalez CF, Lorca GL. *PLoS Pathog.* 2014 Apr 24;10(4):e1004101. doi: 10.1371/journal.ppat.1004101. eCollection 2014 Apr. PMID: 24763829