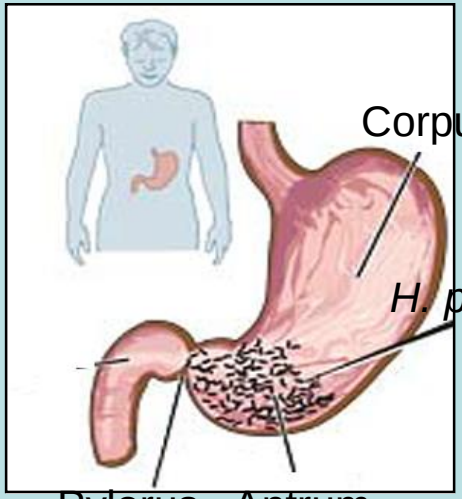
DNA replication in pathogens: Unique properties and possible intervention

Suman Kumar Dhar, Ph. D.
Special Centre for Molecular Medicine
Jawaharlal Nehru University, New Delhi-110067

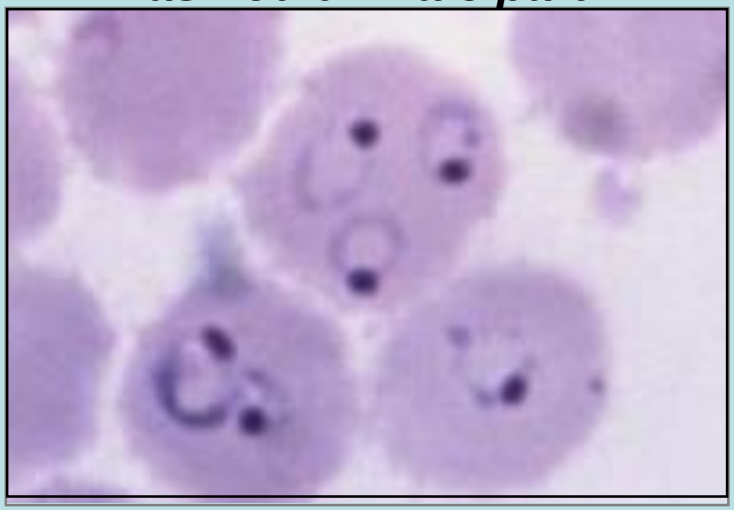
General objectives: Our laboratory focuses on understanding the DNA replication and cell cycle regulation of two medically important human pathogens; *Helicobacter pylori* that infects more than 50% of human population and causes gastric ulcer and gastric adenocarconoma And *Plasmodium falciparum* that causes human malaria.

- **There is no effective vaccine against either of these pathogens**
- **Drug resistance is a serious problem for both of them**

Helicobacter pylori

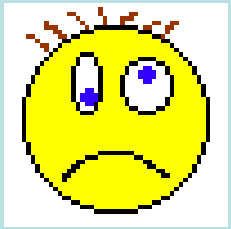


Plasmodium falciparum



Helicobacter pylori

Normal beliefs regarding gastritis



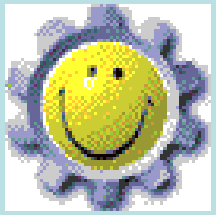
Anxiety
Stress
Irregular food habits
Exam phobia

Gastritis

Acid secretion

Acid blocker; Antacid

Temporary Relief

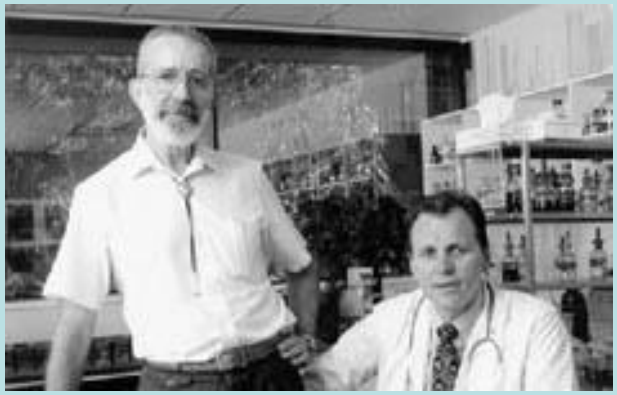


Relapse

Warren and Marshall revolutionised the concept of gastroduodenal diseases

The Nobel Prize in Physiology or Medicine for 2005

Robin observed the presence of small Curved bacteria Colonizing lower part Of the stomach



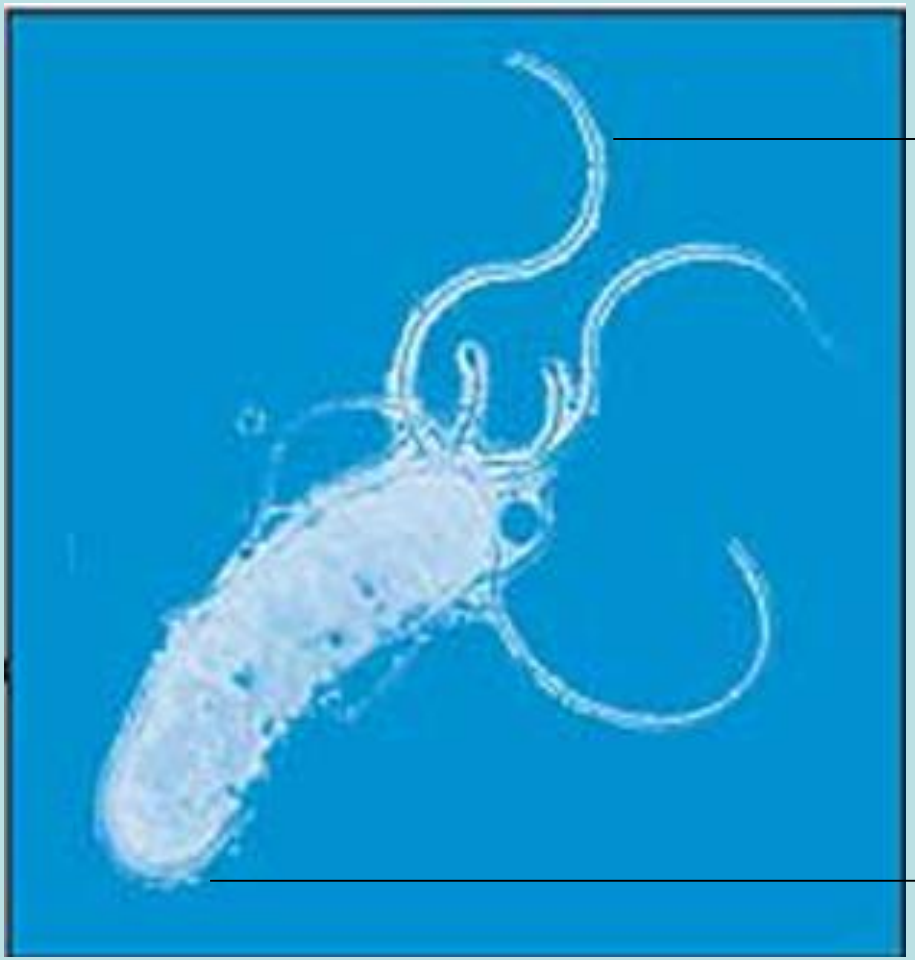
R. Warren B. Marshall

Marshall later Initiated a study With 100 patients & Confirmed the Presence of the Bacteria in the stomach

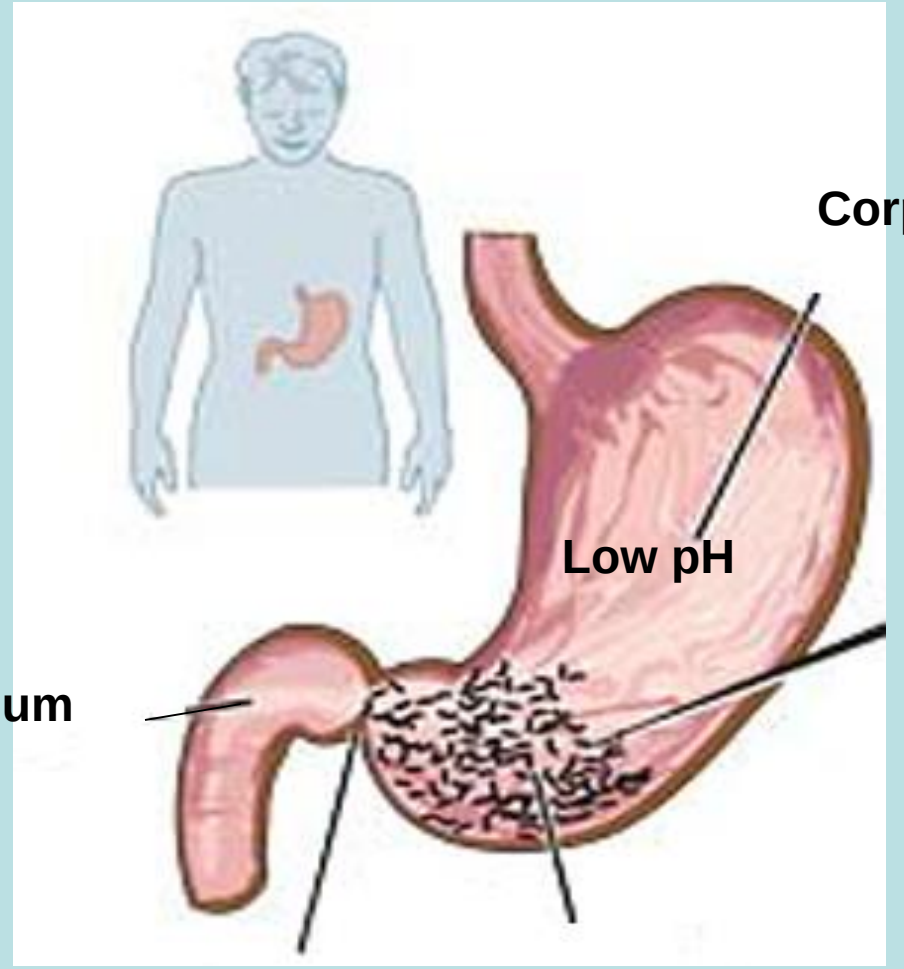
Marshall succeeded in cultivating the bacteria from the biopsies. Together, they found that the organism was present in almost all patients with gastric inflammation, duodenal and gastric ulcer (1982).

The Bacterium

H. pylori

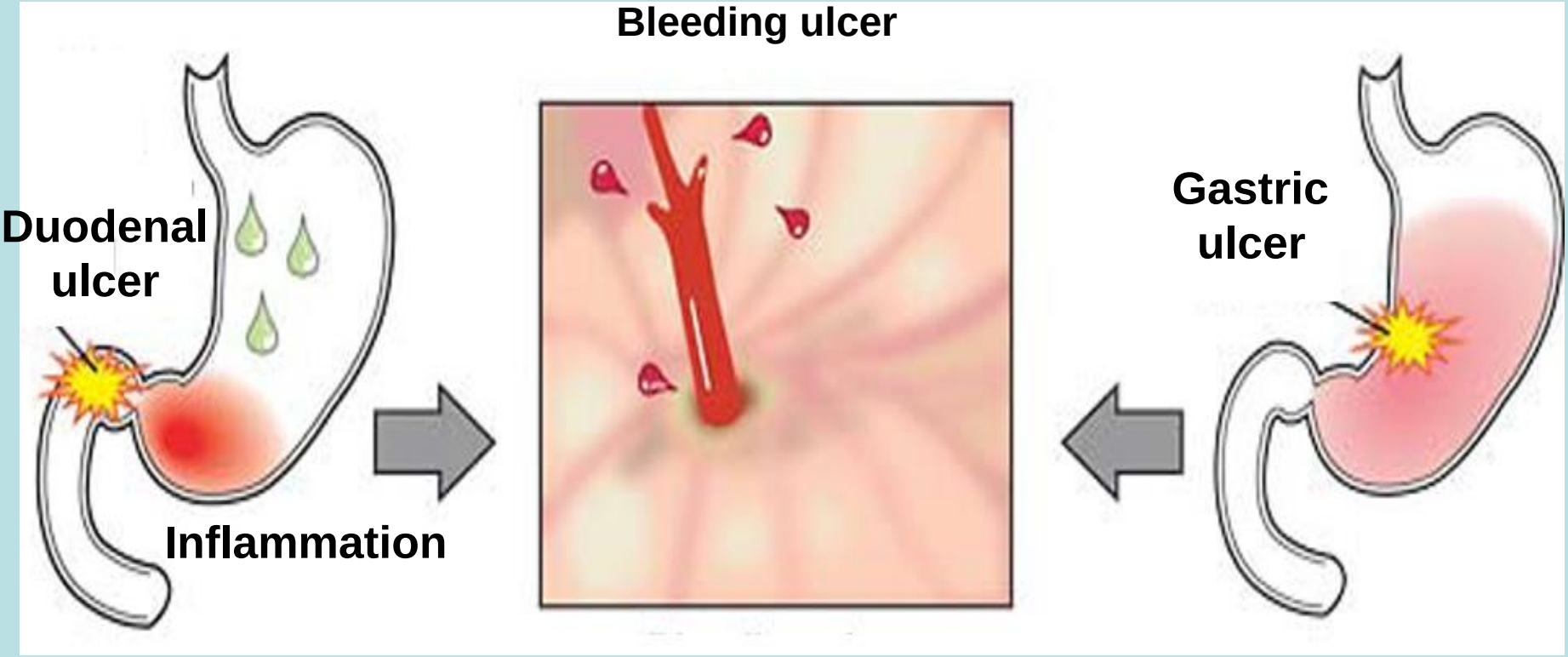


Places of Infection in the stomach



H. pylori infects the lower part of the stomach, antrum

Ulcer (schematic)

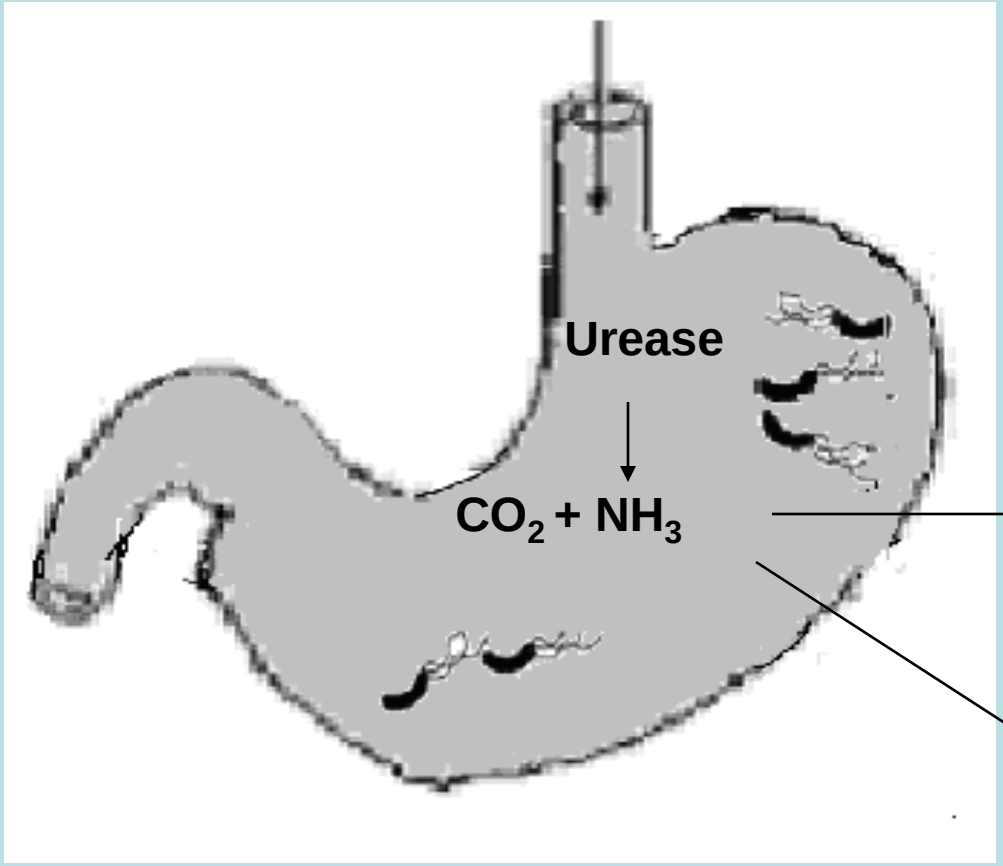


Gastric inflammation may lead to duodenal ulcer followed by bleeding ulcer

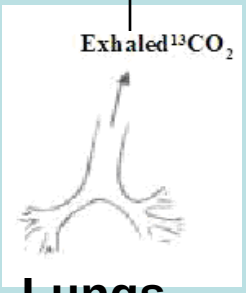
Diagnosis
Bacterial survival and urea breath test for detection

Noninvasive

^{13}C Urea Solution Ingested



$^{13}CO_2$ detected



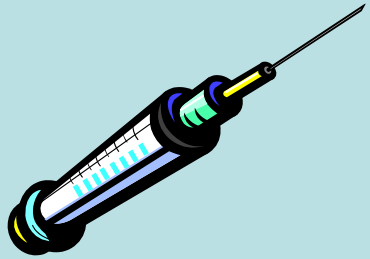
Lungs

$^{13}CO_2$ absorbed in blood

Ammonia Neutralizes Acidic Surroundings

Invasive: Endoscopy → Biopsy → Culture

Treatment



Antibiotics

Amoxycillin, Clarithromycin, Tetracycline and metronidazole.

Acid lowering drugs

Ranitidine, Cimetidine, Famotidine, Omprazole, Pantoprazole and Lansoprazole

Combination of the above two for two weeks is the best strategy

However, there are incidences of drug resistant strains

Vaccination: Not available yet

Malignancies associated with *Helicobacter pylori* infection

***H. pylori* infection in the stomach**

↓

Prolonged and widespread inflammation and gastritis

CagA protein ↓ **??**

Malignant change leads to stomach cancer

Uncontrolled growth of cells

↓

Number two in cancer deaths

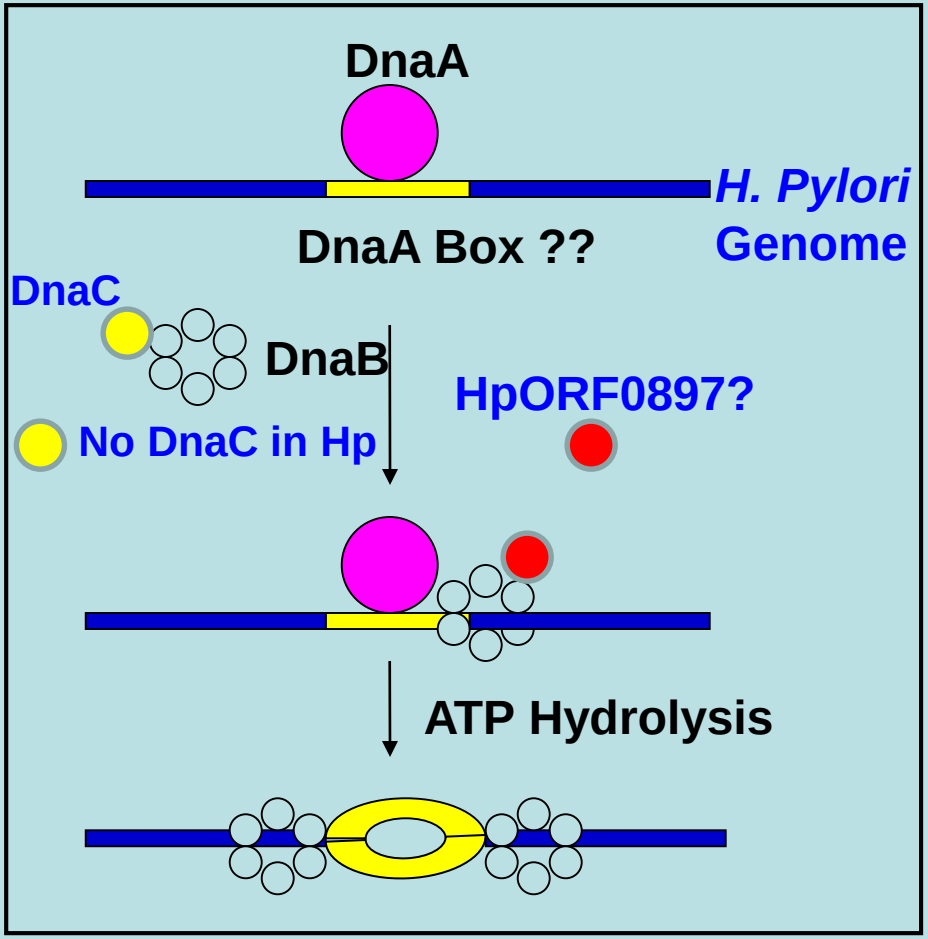
Why to study *H. pylori* DNA replication and cell division?

- **Several virulence factors including CagA, VacA have been reported**
- **Not much is known regarding the basic biology of a slow growing pathogenic bacteria**
- **Analysis of *H. pylori* genomic database reveals some interesting features of the DNA replication initiation machinery and cell division cycle.**
- ***oriC* is not characterized and DnaC which is a helicase loader and essential in other prokaryotes is absent in Hp**
- **It will help us to understand the basic biology of the pathogenic bacteria as well as finding new target(s) for therapy**

Unique features of human pathogenic bacteria *Helicobacter pylori*

DNA replication and cell division cycle

Replication Initiation at *ori*C of *H. pylori*

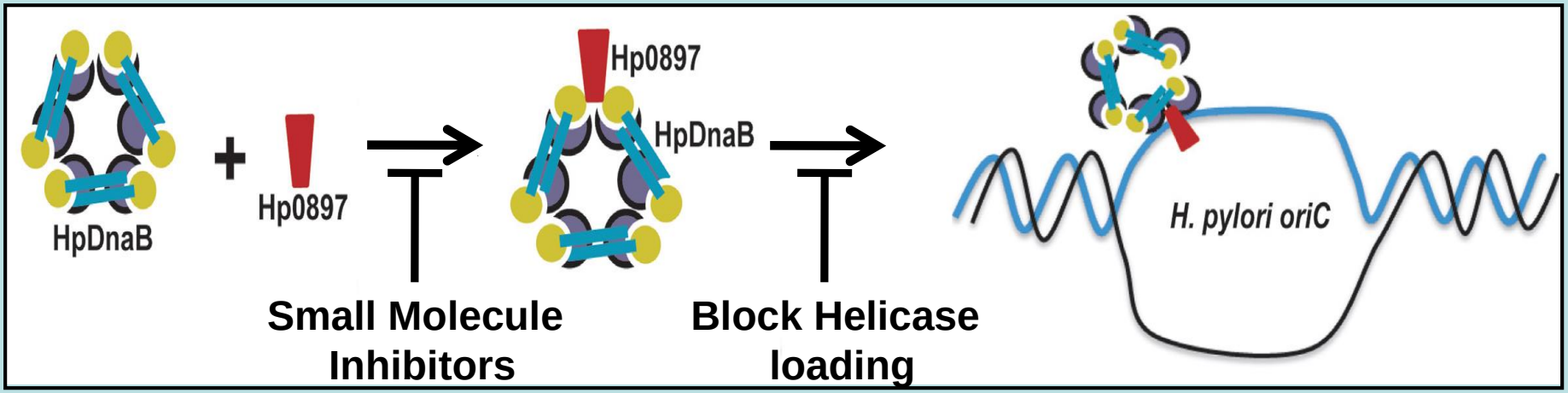


Important findings:

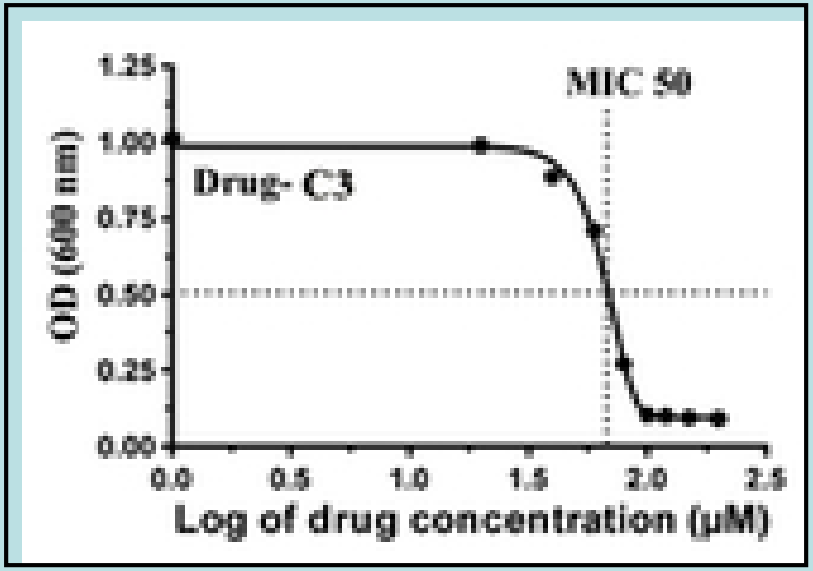
- Identified a unique DnaB Helicase that can work in the absence of Helicase loader DnaC, essential in *E. coli*.
- Identified and characterized an ORF0897 that interacts and modulates the activity of HpDnaB and is linked With Its growth
- *Replication foci is formed at the pole instead of at the middle of the bacteria normally found in other bacteria

Publications: Nucleic Acid Research, 2003, 2007, 2016; Biochem J., 2005; FEBS Lett., 2011, 2017; FEBS J., 2009, 2012; PLoS One, 2009; Journal of Bacteriology, 2013, 2014

Blocking interaction between HpDnaB and Hp0897 may lead to inhibition of bacterial replication and growth



Nucleic Acids Res., 2016

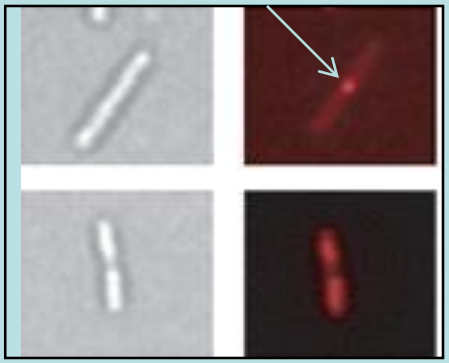


Targeting the β-clamp in *Helicobacter pylori* with FDA-approved drugs reveals micromolar inhibition by diflunisal

FEBS Lett., 2017

Polar replication foci formation and progression of replication foci in *H. pylori*

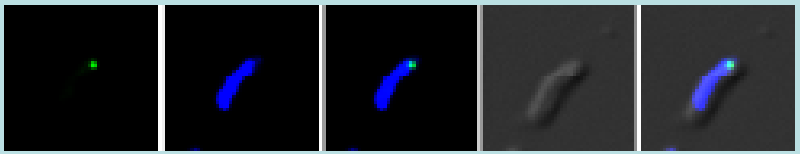
E. coli



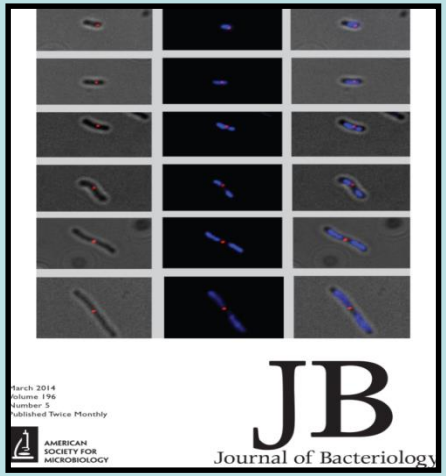
GFP-EcSSB

GFP

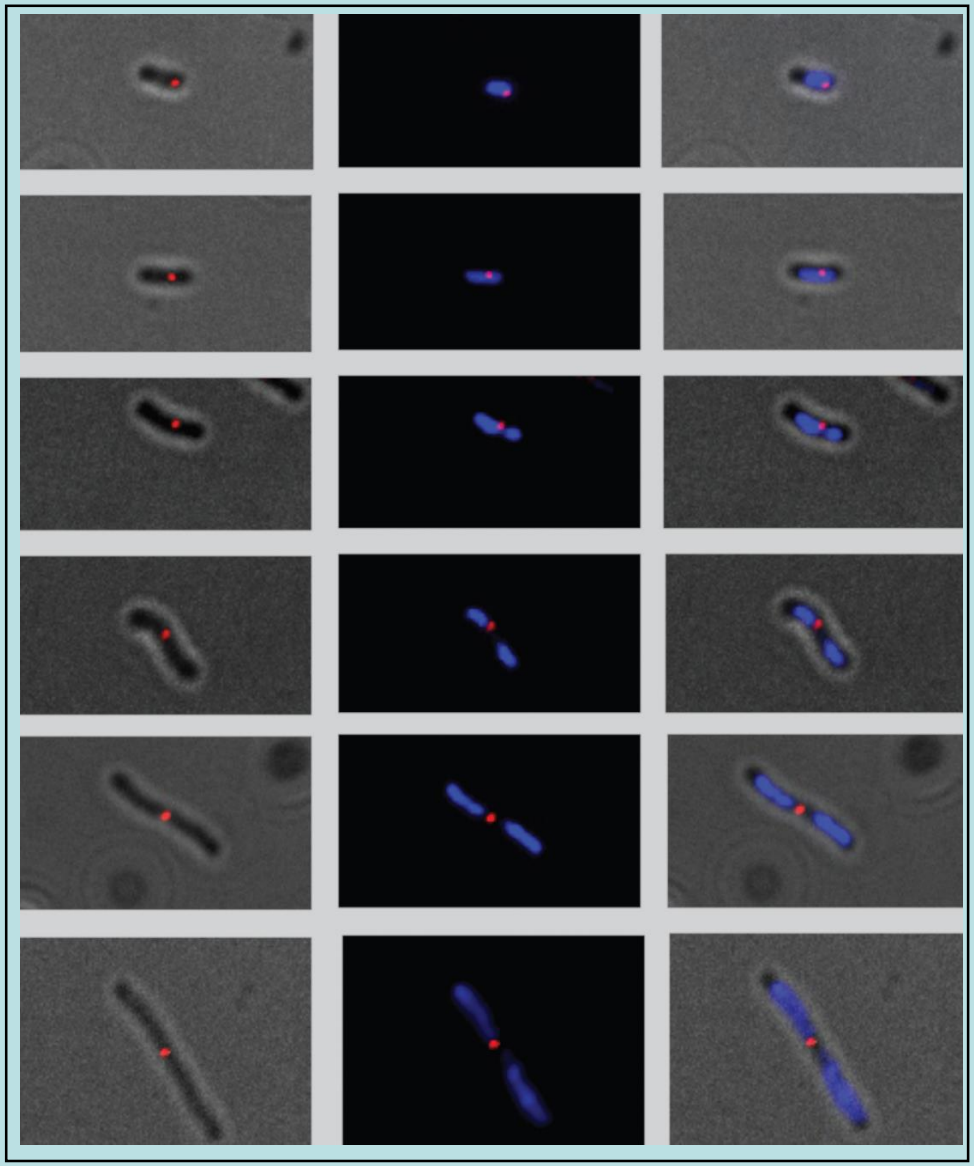
H. pylori



GFP- HpSSB



Sharma A *et al.*,
Journal of Bacteriology,
2014



MALARIA

1. *Plasmodium falciparum* causes more than one million deaths in each year

2. No effective vaccine (100%) is available so far due to antigenic variability

3. High prevalence of conventional drug resistance. Urgent need to identify New targets and novel drugs.

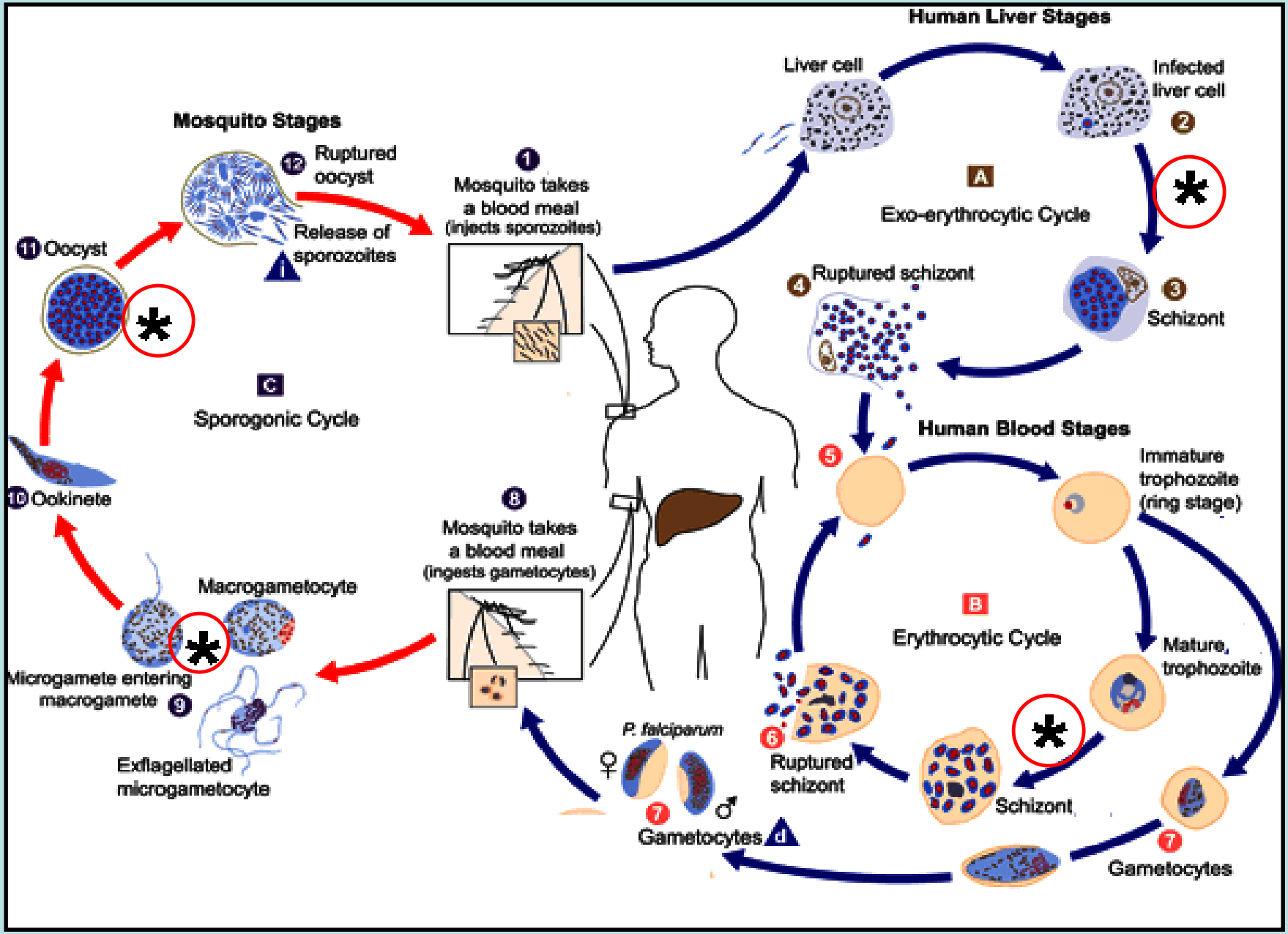
4. Lack of knowledge regarding the fundamental biology and biochemical Processes

5. Understanding DNA replication and related processes could be useful in this regard.

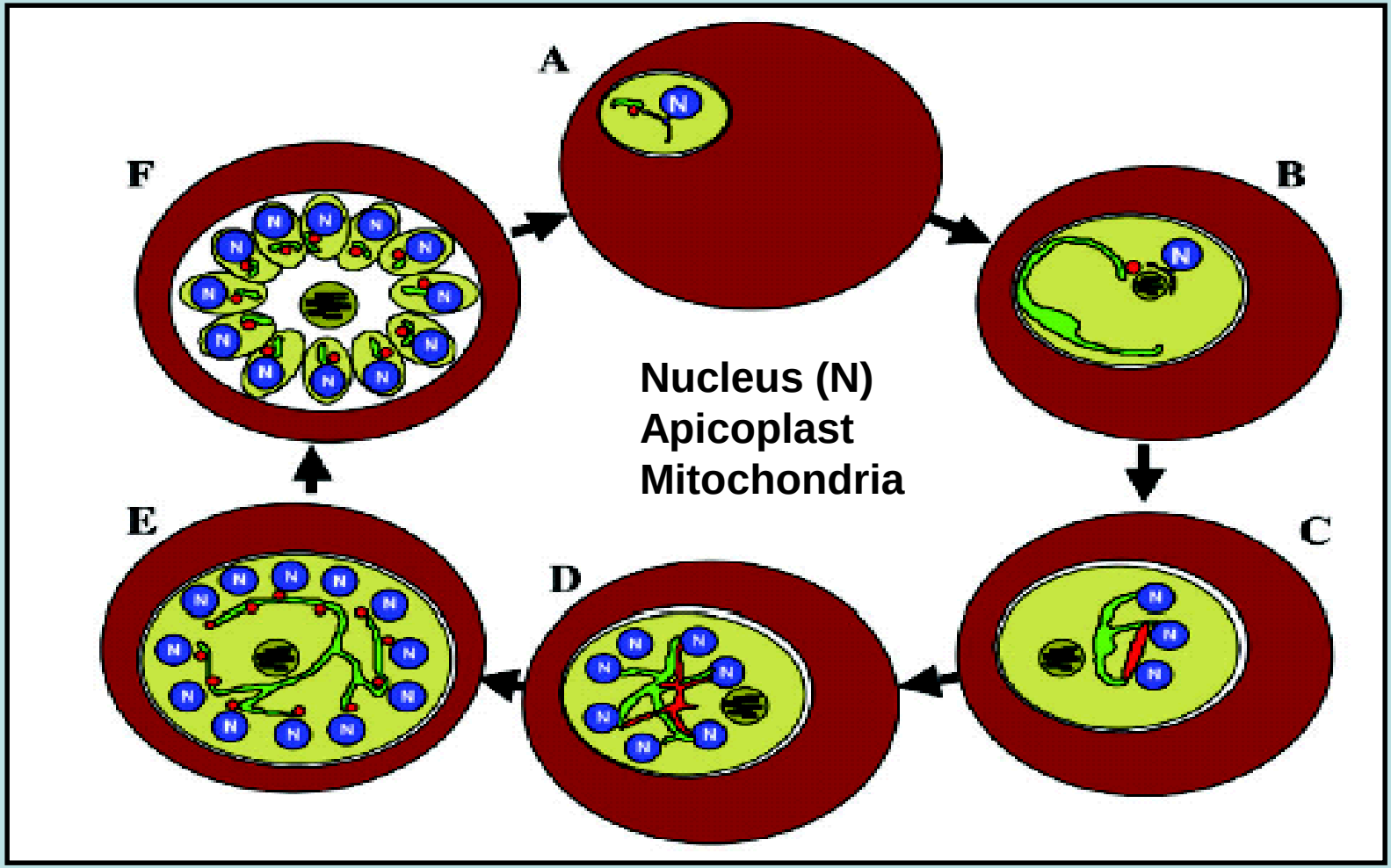
6. DNA replication initiation is the most important rate determining step

7. DNA replication takes place at five distinct points in the parasite life cycle Including the hepatocytic and erythocytic stages.

Life Cycle of *Plasmodium falciparum*



A Simple Model For Nuclear Division, Organelle division and Cytokinesis in *P. falciparum*

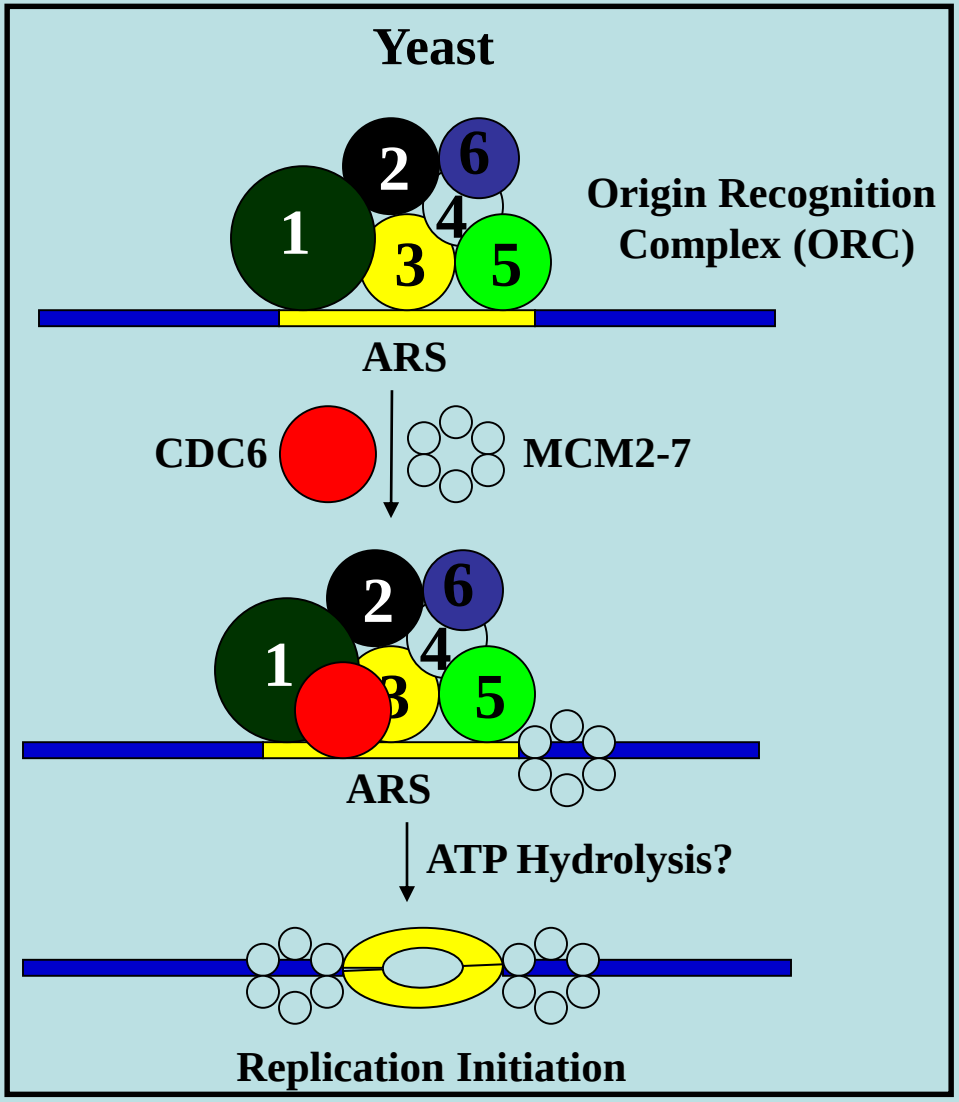


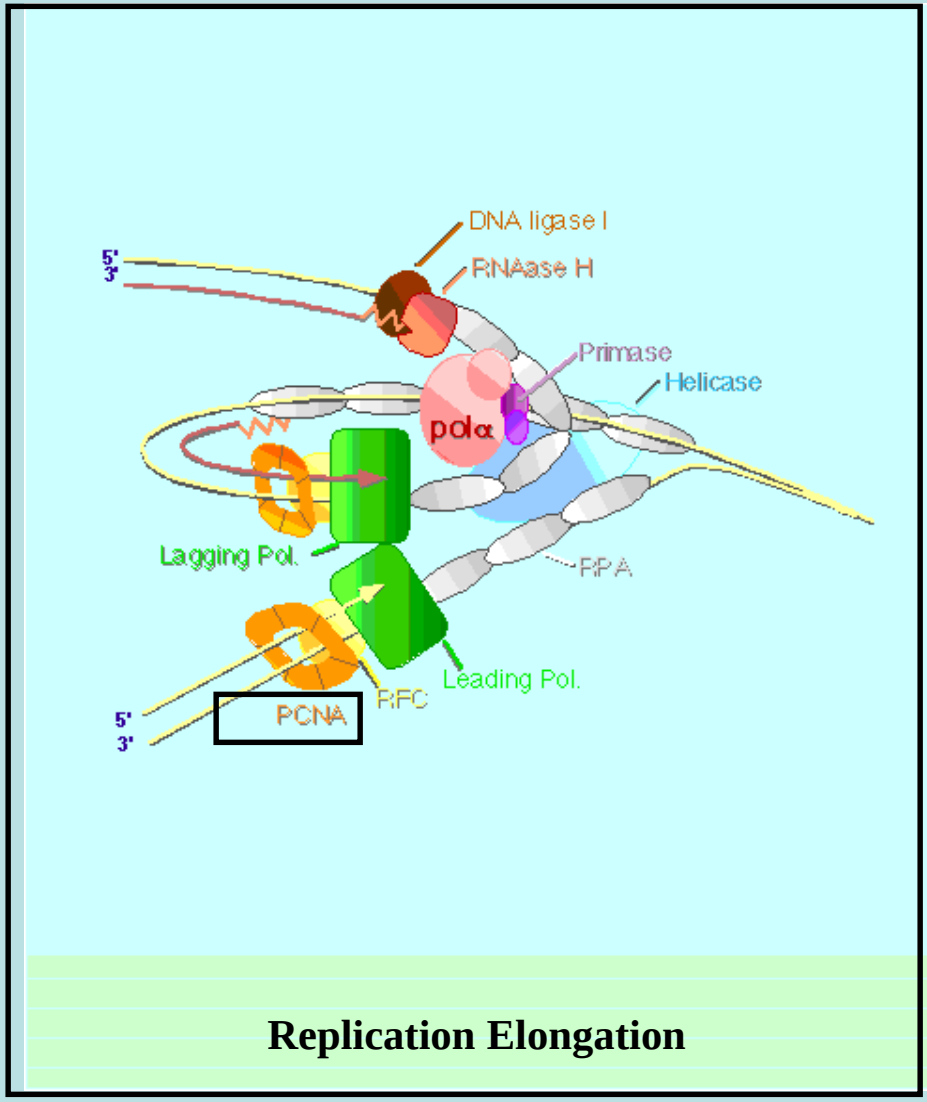
Van Dooren, GG, Mol. Microbiology; 2005

Questions?

- What triggers DNA replication
- How is it regulated
- Where does it happen in the genome
- Can we block DNA replication

Eukaryotic DNA Replication Initiation and Elongation





Is ORC function and cell cycle regulation is conserved in *Plasmodium falciparum?*

Plasmodium falciparum Replication Proteins Identified So Far From The Database:

Chromosomal DNA Replication

1. ORC1 (PFL0150w)
2. ORC2 like protein
3. ORC5 (MAL7P1.21)
4. ORC4 like protein
5. MCM2 (PF14_0177)
6. MCM3 (PFE1345c)
7. MCM4 (PF13_0095)
8. MCM5 (PFL0580w)
9. MCM7 (PF07_0023)

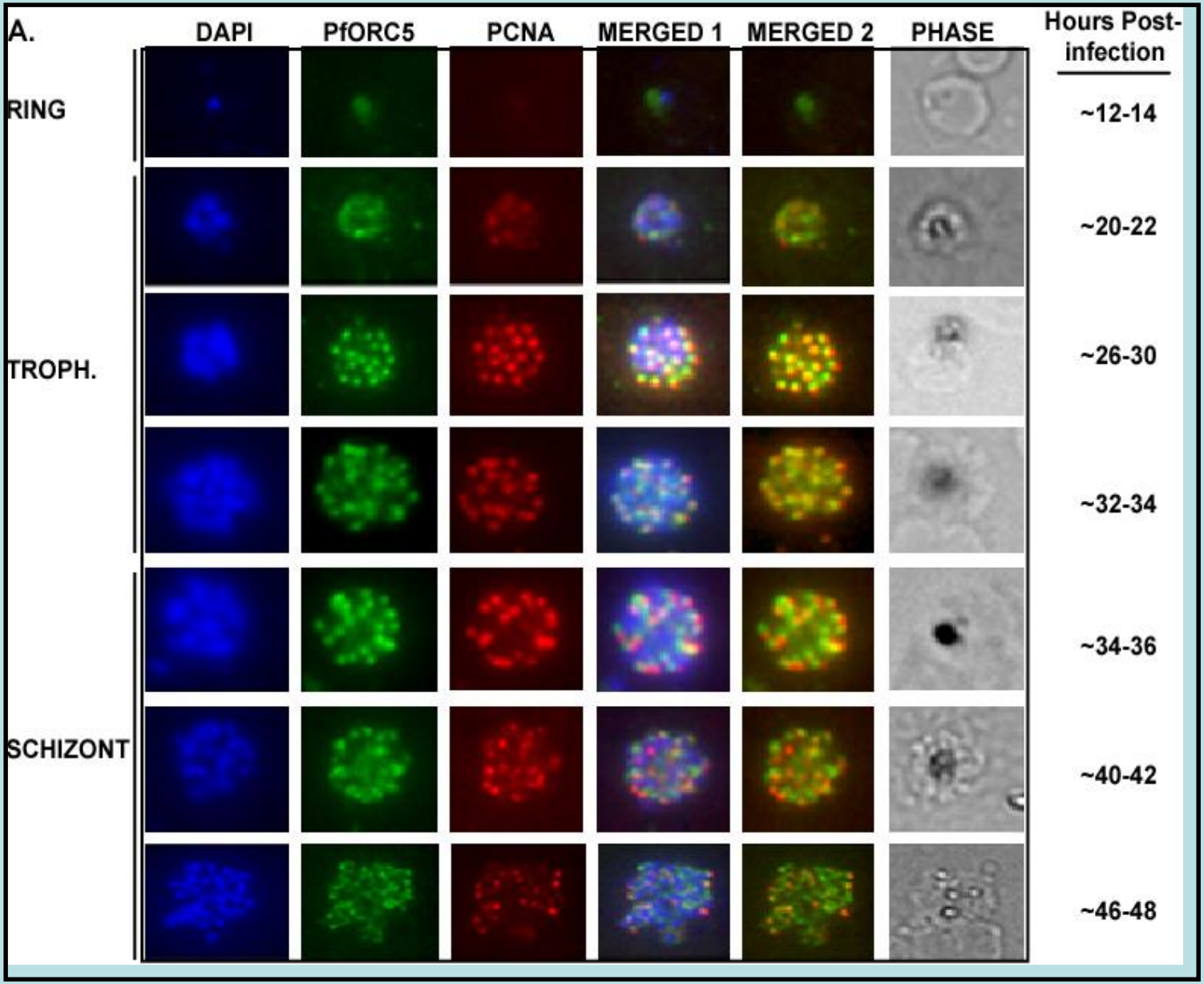
9. PCNA (PFL1285c)

Other Members?

Either there are functional homologs or they are absent.

Other replication proteins identified so far in *Plasmodium falciparum:*
Proliferating cell nuclear antigen (PCNA), DNA polymerase alpha, delta, Replication protein A (RPA)…

DNA replication occurs in discrete foci in *P. falciparum*

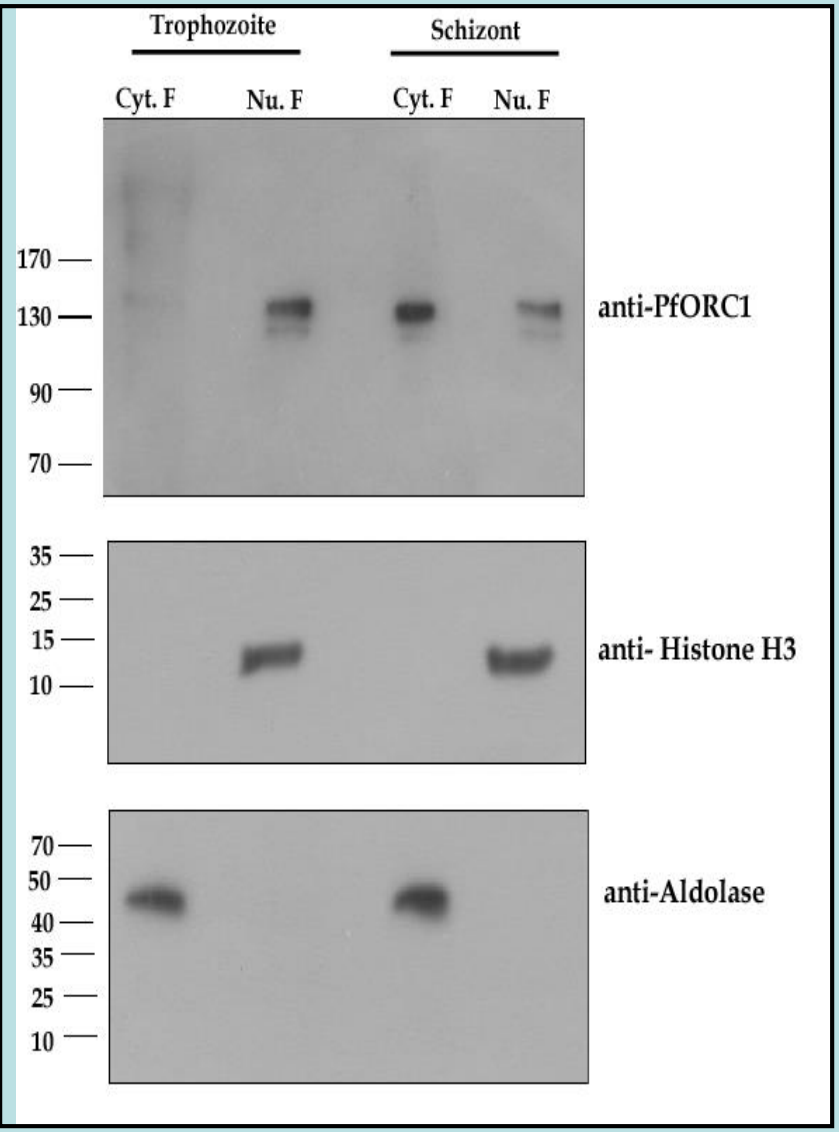


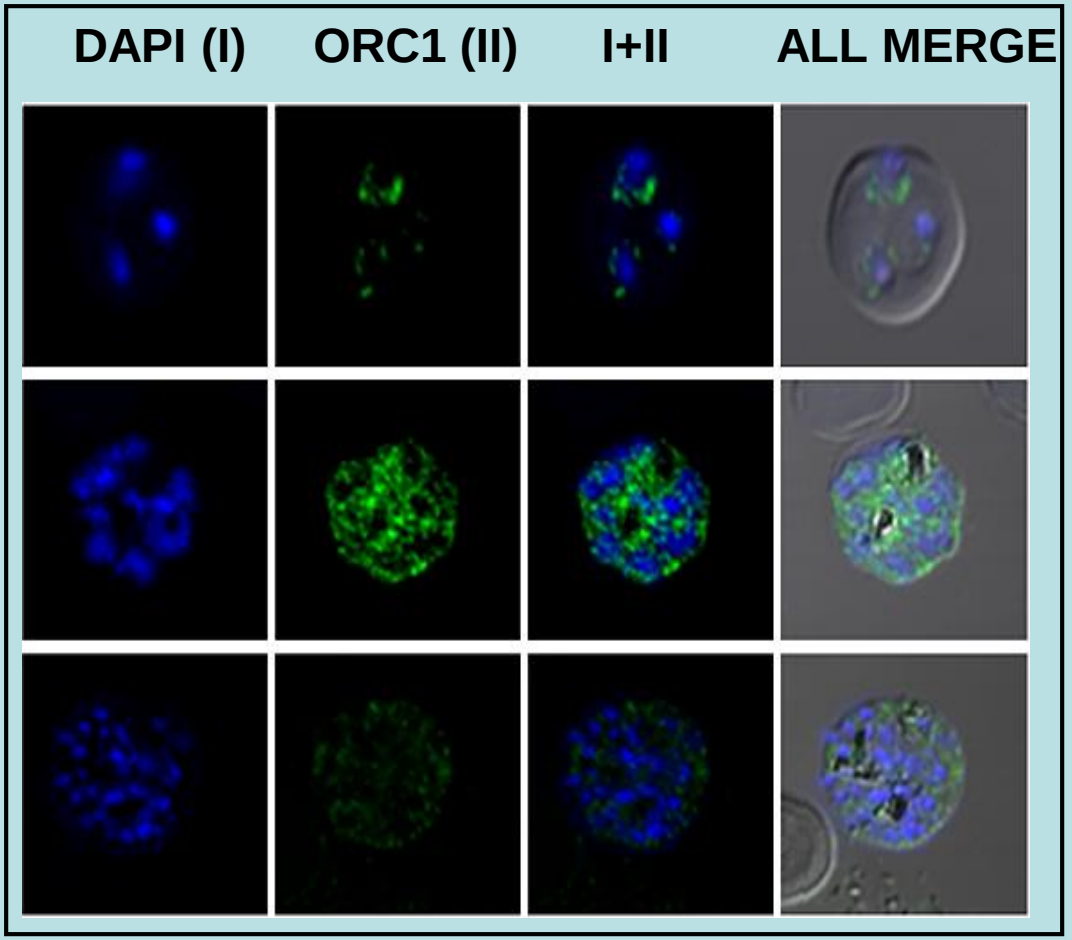
Gupta A et al., Mol. Microbiol. 2008

Regulation.....

Sub-cellular localization of ORC1 through erythrocytic developmental stages

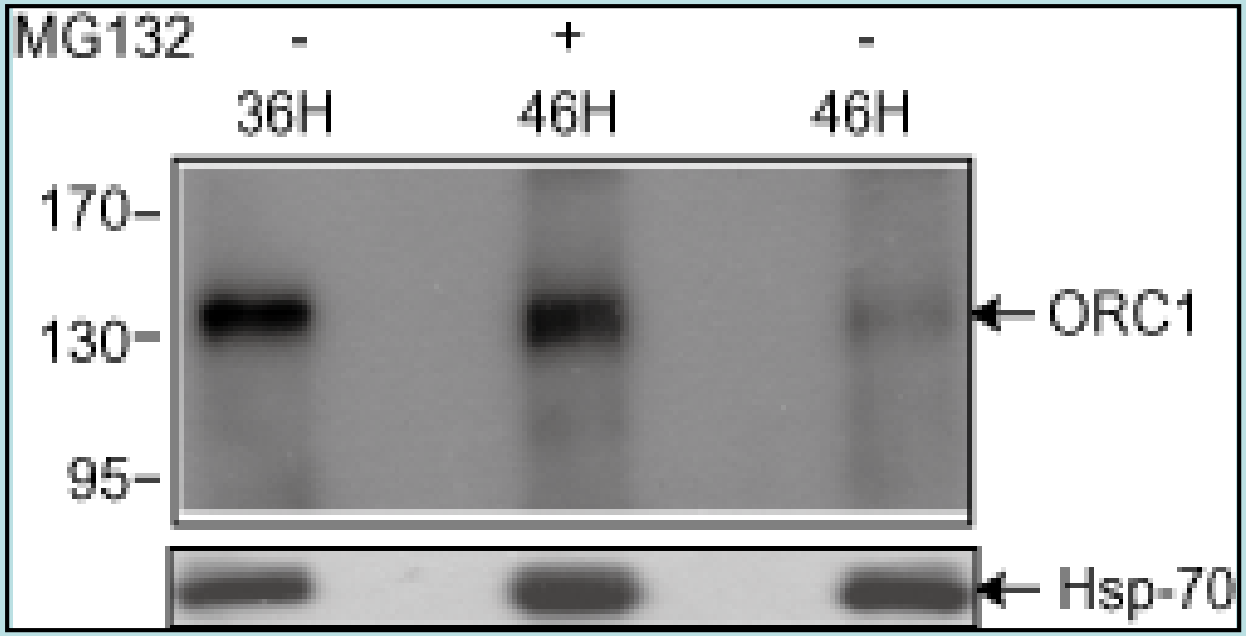
ORC1 shuttles between nucleus and cytoplasm



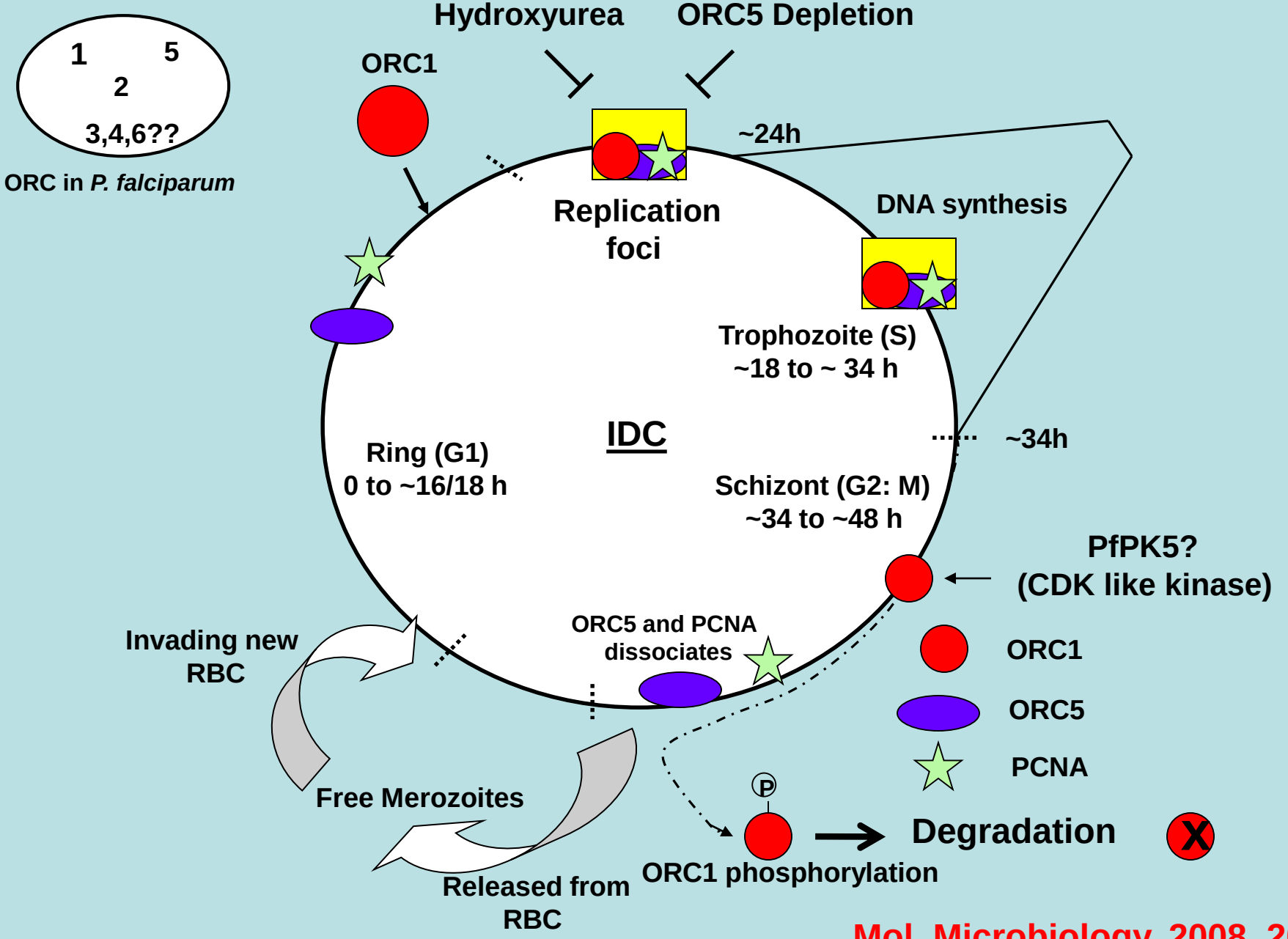


Deshmukh A et al., Mol. Microbiology, 2015

PfORC1 is degraded at the late schizont stage



DNA Replication and cell division cycle in *P. falciparum*



Mol. Microbiology, 2008, 2015

Where Does DNA Replication take place in *P. falciparum* genome?

Autonomous replicating sequences (ARS)

1. **Autonomously replicating sequence** (ARS) are specific origin sequences in budding yeast *Saccharomyces cerevisiae.*

2. ARS contains four regions A, B1, B2 and B3 named in order of their effect on plasmid stability.

3. A elements are called ACS (ARS consensus sequence) has conserved 11bp (A/T)AAA(C/T)ATAAA(A/T).

4. A element with B1 recruits ORC, B2 is required for efficient loading of Mcm2-7 proteins and B3 is binding site for Abf1 transcription factor.

***P. falciparum* genome analysis shows the presence of several ARS like sequences**

Genetic coordinates of PfARS like sequences

1) PfARS Region1- Length of amplified region – 458 bp

>Pf3D7_10 | | 1555756 to 1556214

TATGTATGTACGTATGTATGTATGAATAAAGTAATTTATGTGTATGTAGAAATGAAATAATTAAATT
ATAACTTTCTATAAAATTTTGATGATTAAAAACATCACAGAAAAAAAAAAAAAAAAAAAAAAAAC
ATATATTGAATAAAATATAGGTAATAATTATTTCAAATTTTTTATAATTTAAATGACATAATTAAAAA
GGATACAATAAAATAAATCAAAAAAGAAACAATGTTATATATTTTTTTGTTTATTTATTTATTTATTT
ATATTTAAAATTTATTTTATATTTTTAATTAAATAAAGATTTATATATCCTATAAATTTTGTAATATAT
AAAAATTTCAATTGATTCATTGTTATTTTAAAATATGTATTCTTCTCACCAAATATAAAAAAAAAAA
AATTAAAATTAACTTAATGTGAATATGATTTGAGAATTTAAGTCACTACATATA

2) PfARS3 Region - Length of amplified region – 499 bp

>Pf3D7_10 | | 1517716 to 1518215

ATACCTAATAATTCAGACAAAATATAGATATGAATAATATTTATAATTTTTATGTGTTTTTA
TATTTTTTTTTATATTTTATTCATTTCATTAATATATATACTATTGTTTTTATTTATTAATAGA
AATAAAAGAAAAATAGGTATAATAAAAAATTTGGTATTTTAATATTTCTCATTATAAATAA
ATAAATGTATATATATATATATATATATATGTATATTTCTTTATATTATTTTGAAAAATTAAT
ATCTTTCTTTTTGAATGACTTATATTACATAATACATGATGTTATATACATAATATAGGAT
ATTGATATATGTAGATGTGTTTTGTATATATGAGACAAATCTTATATTTTCTTTAATTTTTT
TTTTCTTTTTTCTTTTTTCTTTTTTTTTTTTTTTTTTTTTATATATCTTATAAGTTATTTCTAATA
TATTAGAAAAATAATTGACATTTTTTTTTGAATTATATATAGAAAGTTATATTGTTTCATTAT
G

Controls: AT-Rich, GC-Rich sequences and Delta ARS3 sequences

FEBS Journal, 2017

Yeast transformation assay to confirm ARS like elements

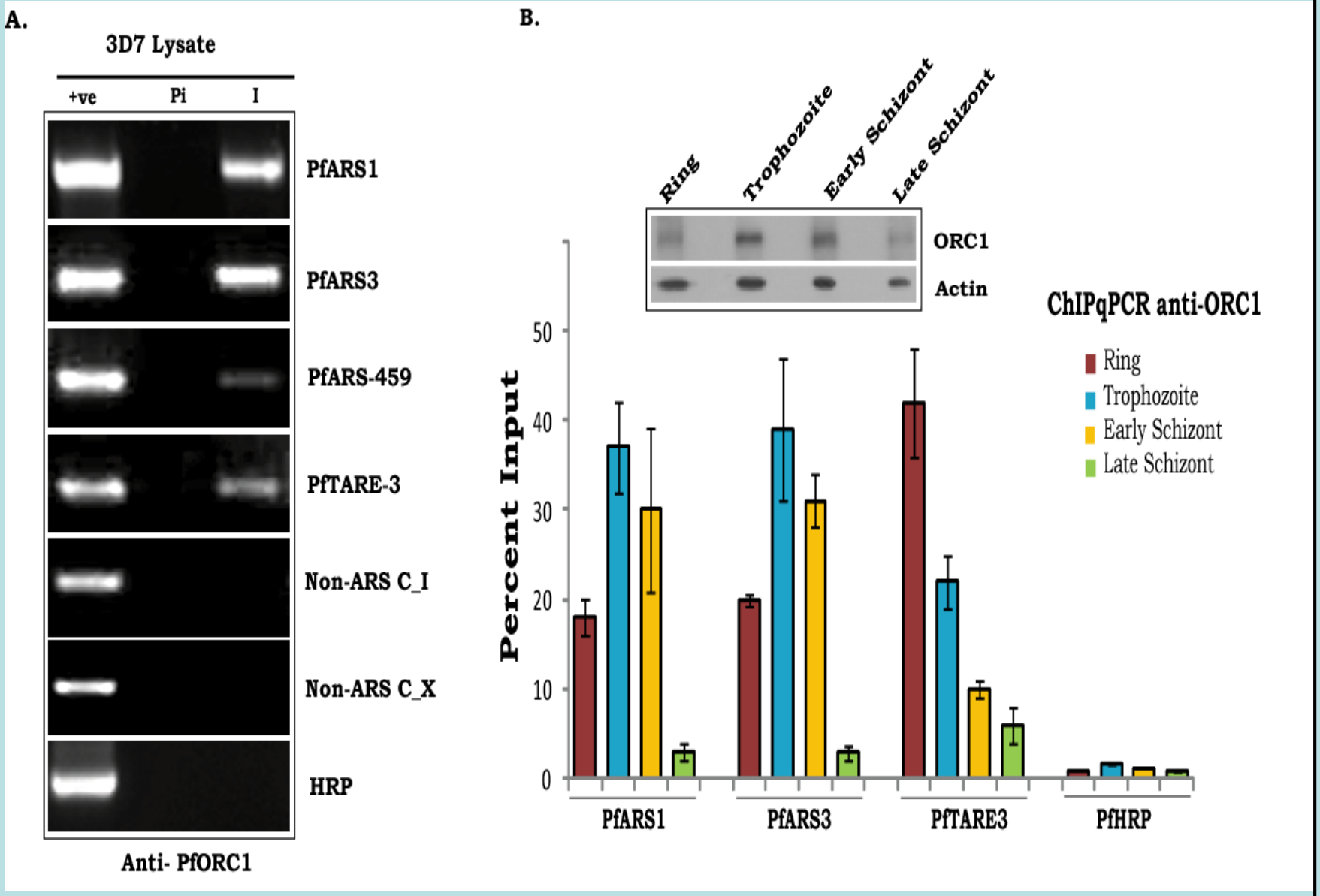
Transformation Assay

Transformed plasmid	Transformation 1	Transformation 2	Transformation 3	Average
ScARS	420	480	564	488
PfARS1	340	263	228	277
PfARS3	375	346	388	370
PfAT rich	-	-	-	-
PfGC rich	-	3	4	2
PfARS3Δ	-	2	-	0.66
ARS(-)ve	-	4	6	3.33

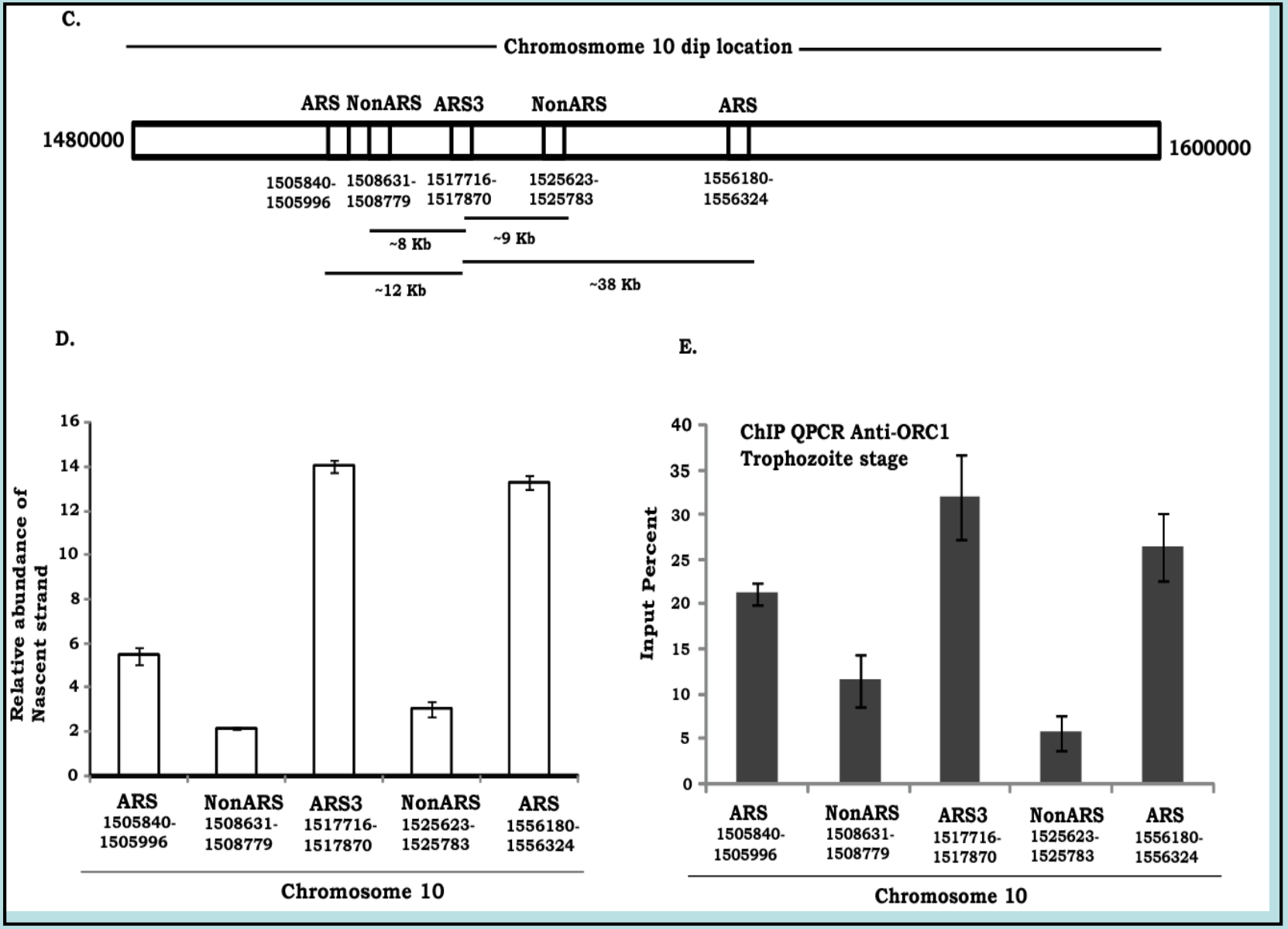
Plasmid Stability Assay

Plasmid	Trans. Frequency	% cells having plasmid	% loss per Gen.
ScARS	488	98 ± 0.1 - 0.9%	2.25 – 3.66% (n = 6-12)
PfARS1	277	70 ± 25%	19.1 – 26 % (n = 6-12)
PfARS3	370	65 ± 25%	23.25 - 26.5 % (n = 6-12)

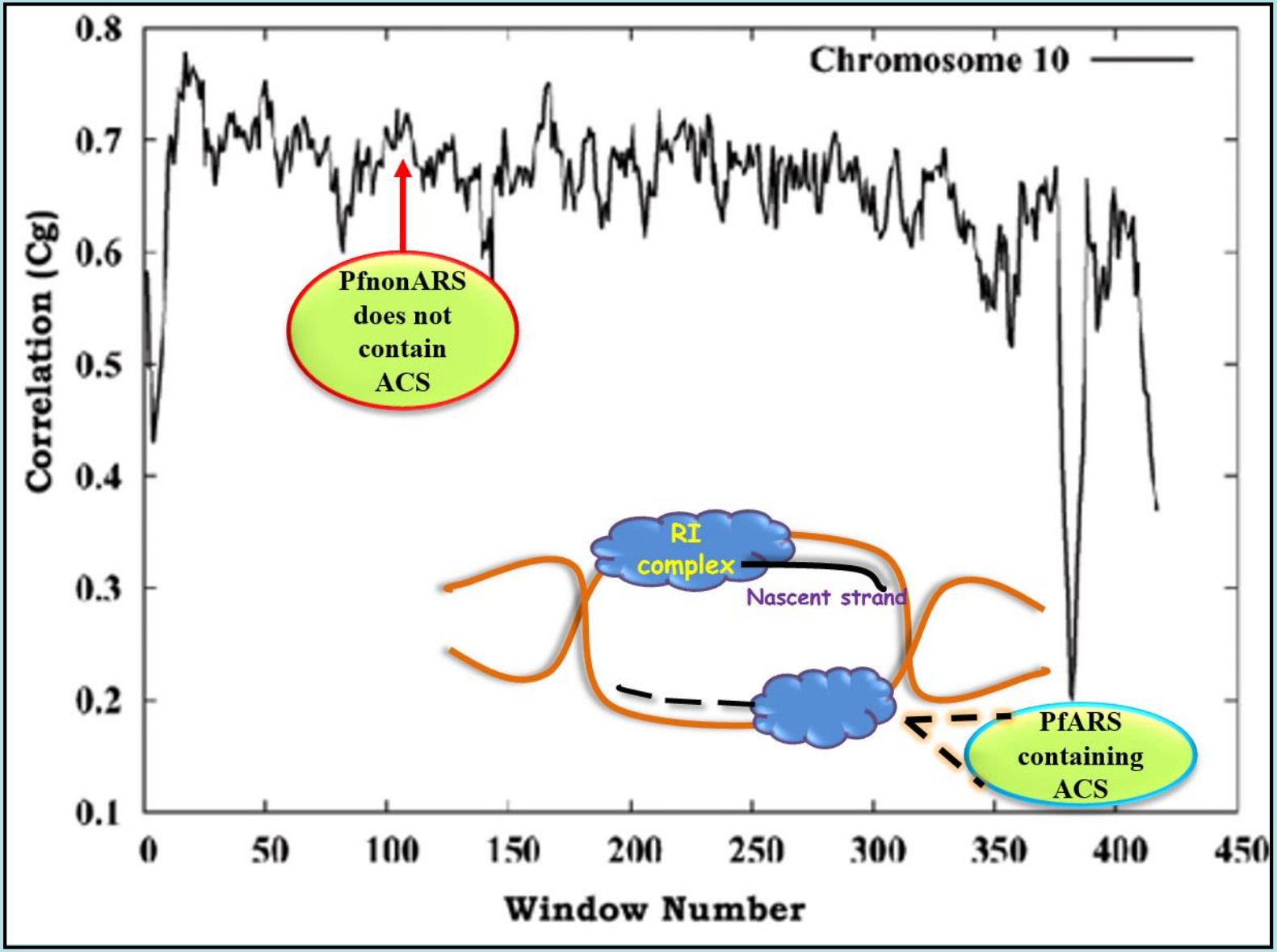
Endogenous PfORC1 binds to PfARS sequences in *Plasmodium falciparum* and the binding is maximum during replicating trophozoite stage



ORC binding and nascent strand abundance is found specifically around ARS elements



Autocorrelation method shows the presence of dip region with the presence of ARS elements as potential origins



FEBS Journal, 2017

Application…..

PfARS3 sequence provides more stability to plasmid DNA compared to non-ARS sequence.

A.

Pf ARS3

Transfected parasite lines (PfARS3/PfnonARS)

Pf Non ARS

WR drug selection →

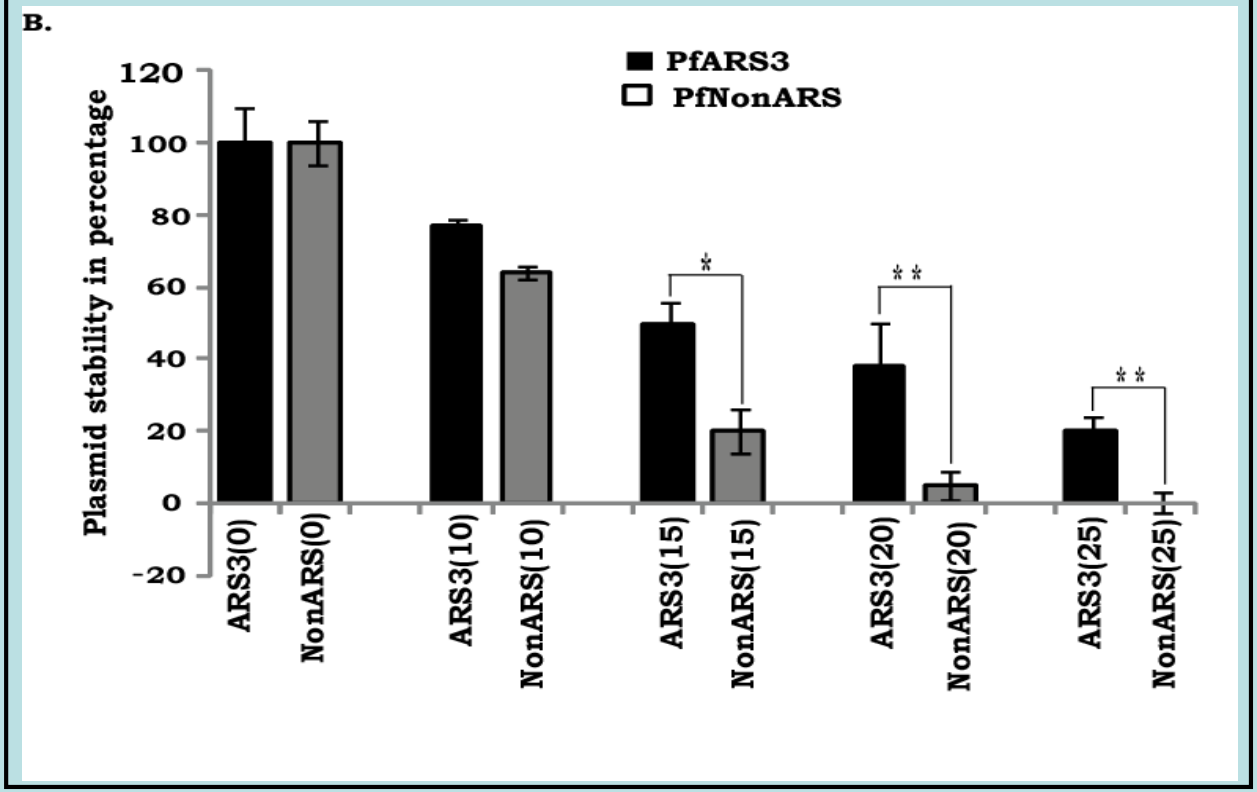
Parasite under selection pressure **(Zero time point)**

Release drug pressure →

Harvest parasites after 10^{th}, 15^{th},20^{th} and 25^{th} cycle →

Quantification of plasmid copy number using qRT PCR

B.

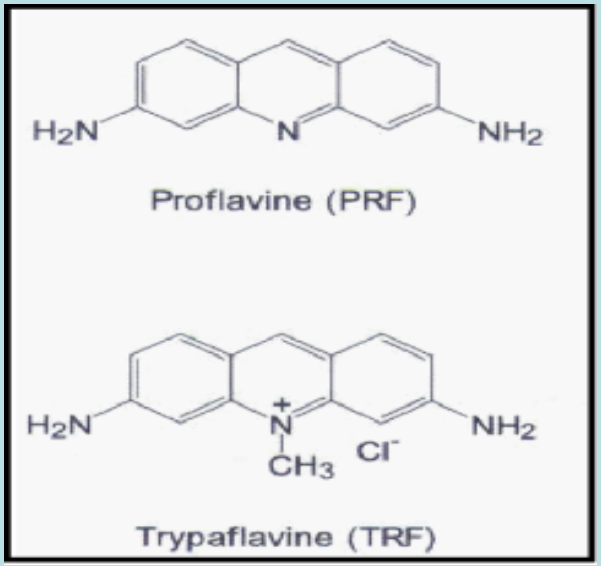


Generation of transfection vector with better stability

Application…..

Can we target DNA replication in the parasites?

To study the potential of ACF as an anti-malarial



Acriflavine (ACF) is a mixture of Proflavine and Trypaflavine

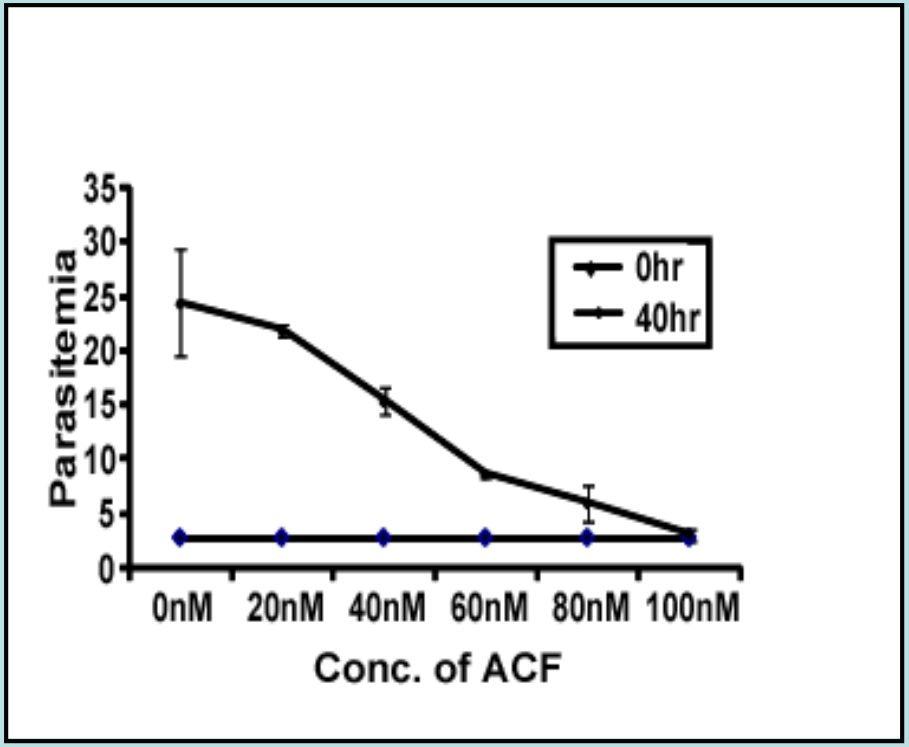
Rationale to use ACF as anti-malarial:

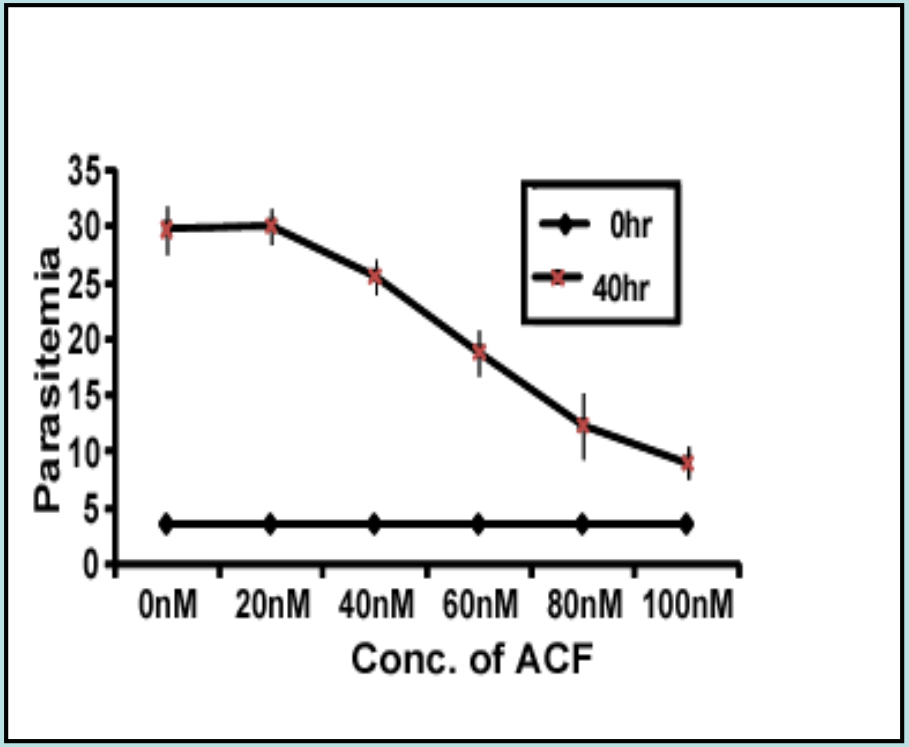
ACF has been shown to have potential anti-cancer activity in mice (Lee K et al., 2009) and it is FDA approved drug with no or minimal toxicity

ACF is an anti-bacterial acridine and it has been used widely as antiseptic (Browning, 1922)

ACF was used as antiparasitic agent during World War II

ACF shows potent anti-plasmodial activity *in vitro*





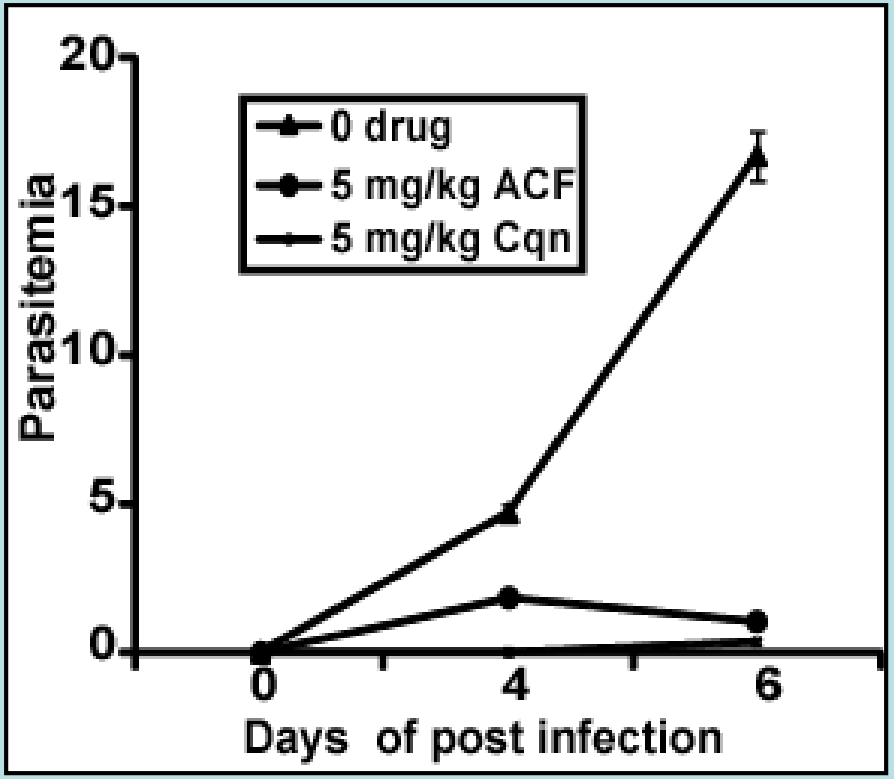
Chloroquine sensitive strain **Chloroquine resistant strain**

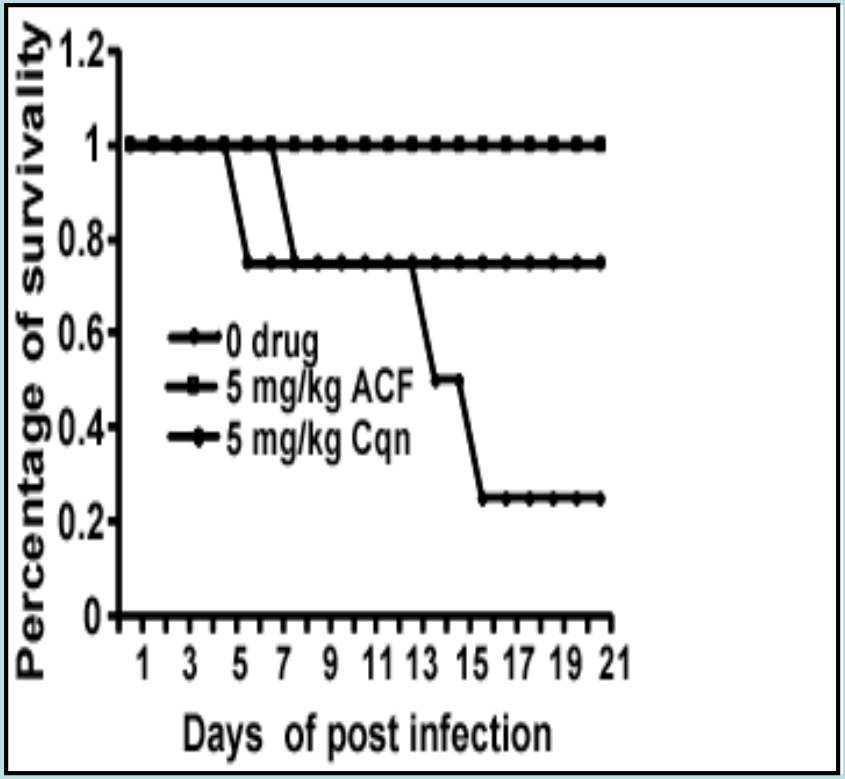
IC_{50}~50 nM

<u>Trypaflavine</u> not proflavine shows anti-plasmodial activity

ACS Chemical Biology, 2015

ACF shows potent anti-plasmodial activity *in vivo*



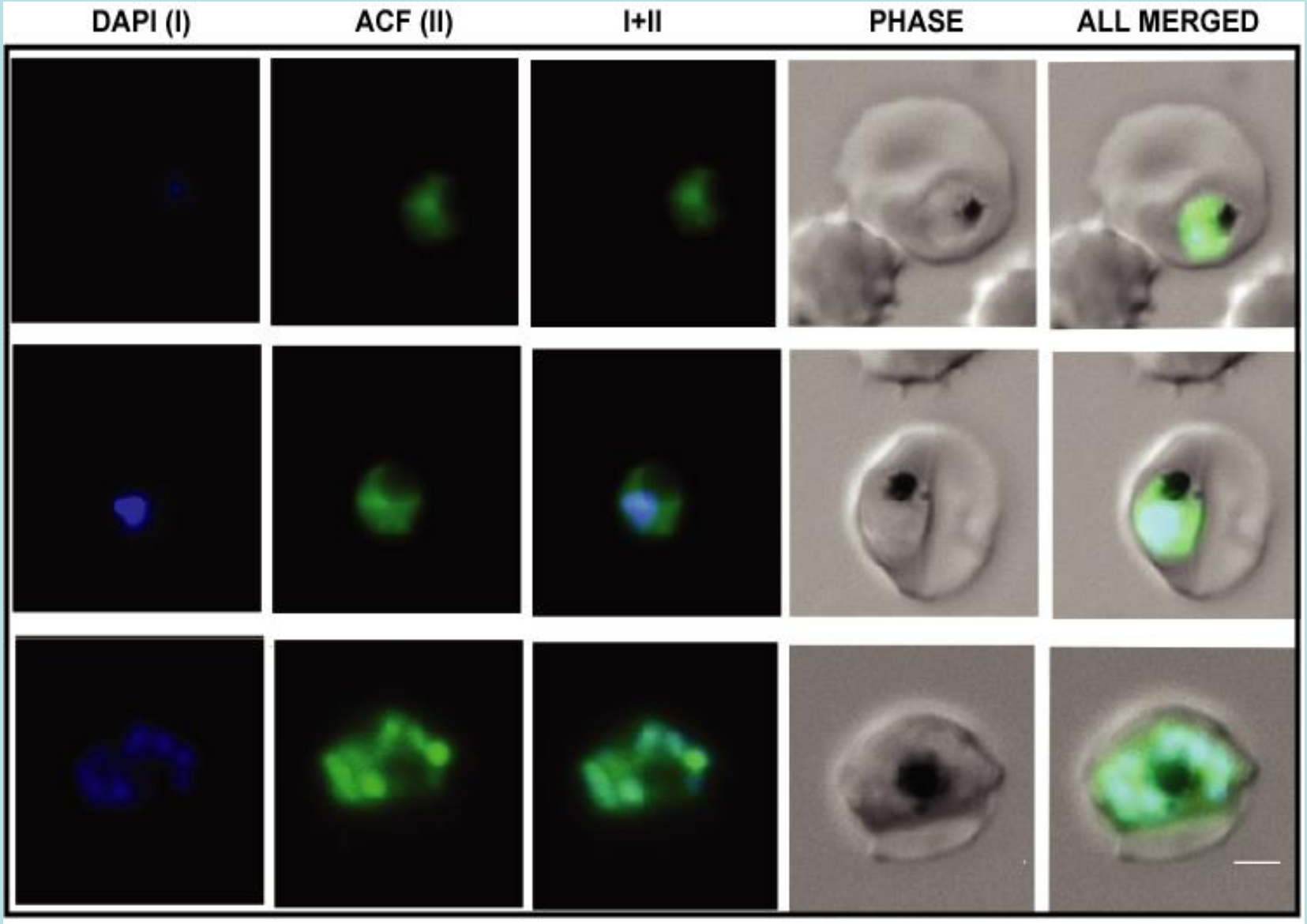


Parasitemia

Survivality

ACS Chemical Biology, 2015
US patent 2016

ACF is accumulated in the parasite infected RBC only



US009375426B2

(12) **United States Patent**
Dhar et al.

(10) **Patent No.: US 9,375,426 B2**
(45) **Date of Patent: Jun. 28, 2016**

(54) **METHOD OF SCREENING ANTI-PLASMODIAL ACTIVITY OF ACRIFLAVIN AND ACRIFLAVIN AS AN ANTI-MALARIAL AGENT**

(71) Applicant: **Suman Kumar Dhar**, New Dehli (IN)

(72) Inventors: **Suman Kumar Dhar**, New Dehli (IN); **Srikanta Dana**, New Dehli (IN); **Ashraf Dar**, New Dehli (IN); **Dhaneswar Prusty**, New Dehli (IN); **Pritam Mukhopadhyay**, New Dehli (IN)

(73) Assignee: **Suman Kumar Dhar**, New Dehli (IN)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **14/423,477**

(22) PCT Filed: **Jul. 4, 2013**

(86) PCT No.: **PCT/IN2013/000411**

§ 371 (c)(1),
(2) Date: **Feb. 24, 2015**

(87) PCT Pub. No.: **WO2014/030171**

PCT Pub. Date: **Feb. 27, 2013**

(65) **Prior Publication Data**

US 2015/0216853 A1 Aug. 6, 2015

(30) **Foreign Application Priority Data**

Aug. 24, 2012 (IN) 2630/DEL/2012

(51) **Int. Cl.**

A61K 31/42	(2006.01)
A61K 31/473	(2006.01)
G01N 33/569	(2006.01)
C12Q 1/18	(2006.01)
C12Q 1/533	(2006.01)

(52) **U.S. Cl.**

CPC ***A61K 31/473*** (2013.01); ***C12Q 1/18*** (2013.01); ***C12Q 1/533*** (2013.01); ***G01N 33/56905*** (2013.01); *G01N 2333/445* (2013.01); *G01N 2500/02* (2013.01)

(58) **Field of Classification Search**

CPC A61K 31/473; C12Q 1/533; C12Q 1/18; G01N 33/56905

USPC 514/380

See application file for complete search history.

(56) **References Cited**

U.S. PATENT DOCUMENTS

2012/0172292 A1 7/2012 Nudler et al.

FOREIGN PATENT DOCUMENTS

WO 0003751 1/2000

OTHER PUBLICATIONS

Weisman et al ,Chemical Biology &Drug Design , Jun. 2006, 67(6), p. 406–409.*

(Continued)

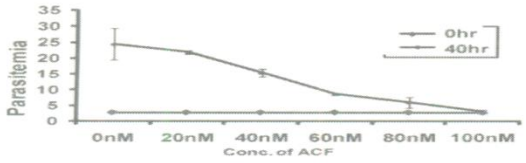
Primary Examiner — T. Victor Oh

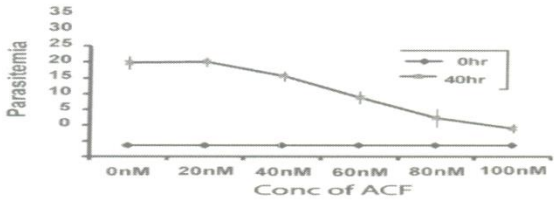
(74) *Attorney, Agent, or Firm* — Renner Kenner Greive Bobak Taylor Weber

(57) **ABSTRACT**

The present invention provides a method of screening anti-plasmodial activity of Acriflavin, comprising assessing growth inhibition of *plasmodium* in vitro in chloroquine susceptible and cloroquine resistant *plasmodium* by Acriflavin; or measuring in-vivo *plasmodium* killing ability of Acriflavin; assessing localization of Acriflavin at different stages; and analyzing effect of Acriflavin on gyrase activity wherein said method utilizes Acriflavin in nano-molar range. The present invention relates to potency of Acriflavin (Acriflavin) as an anti-malarial agent both in vitro parasite culture as well as in vivo. More specifically, the invention relates to a method o determining anti-plasmodial activity, Acriflavin as potent anti-malarial agent and also relates to composition(s) comprising Acriflavin.

14 Claims, 4 Drawing Sheets





Future Plans

- **PfORC1 is the key molecule for the regulation of DNA replication in the parasites.**

- **The presence of ARS like sequences in *Plasmodium* genome is intriguing. We are investigating the presence of ARS like sequences in other *Plasmodium* chromosomes.**

- **ORC binding is a characteristic feature of origin so we would check *Plasmodium* ORC binding on all PfARS sequences.**

- **We will perform ChIP-seq experiments to find out global ORC1 binding sites in the *Plasmodium* genome.**

- **ACF is a potent anti-malarial both *in vitro* and *in vivo***

- **It is possible to design *Plasmodium* specific molecules for anti-parasitic activities**

Acknowledgements

SCMM, JNU
Ashish Gupta
Parul Mehra
Abhijeet Deshmukh
Rahul Sharma
Srikanta Dana
Meetu Agarwal
Kamran Mohammed

Research Funding:
DST, India
DBT(CoE), India
UGC, India
ICMR, India

Dr Tim Gilberger, Germany; Dr T. Adak, NIMR, Delhi; Dr A Krishnamachari, JNU;
Dr K. Shah, IIT, Delhi, Prof. T. Kundu, JNCSAR, Bangalore