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Genomic instability associated with formation of RNA:DNA hybrids and molecular mechanisms of its suppression

Genomová nestabilita spojená se vznikem RNA:DNA hybridů a mechanismy jejího potlačení

Doctoral Thesis

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Čestné prohlášení

Čestně prohlašuji, že jsem předloženou práci vypracovala samostatně s použitím uvedených informačních zdrojů a literatury, s odbornou pomocí vedoucího RNDr. Jany Dobrovolné, PhD a odborného konzultanta Dr. Pavla Janščáka, PhD. Rovněž prohlašuji, že předložená práce ani její podstatná část nebyla použita k získání jiného nebo stejného akademického titulu.

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LIST OF ABBREVIATIONS

ADP	Adenosine 5'-diphosphate
ADP-heptose	ADP-L-glycero-β-D-manno-heptose
AGS	Adenocarcinoma-derived gastric epithelial cells
AGs	Aicardi-Goutières syndrome
AID	Activation-induced deaminase
ALPK1	Alpha-protein kinase 1
AMP	Adenosine monophosphate
APCs	Antigen-presenting cells
APE1	Apurinic/apyrimidinic endonuclease
AQR	Aquarius
ASF1	Alternative splicing factor 1
ATP	Adenosine 5'-triphosphate
ATR	Ataxia telangiectasia and Rad3-related protein
BabA	Blood group antigen-binding adhesion protein
BER	Base excision repair
BLM	Bloom Syndrome DNA helicase
BRCA1	Breast cancer type 1 susceptibility protein
BRCA2	Breast cancer type 2 susceptibility protein
CagA	Cytotoxin-associated gene A
cagPAI	Cytotoxin-associated genes pathogenicity island
CFS	Common fragile site
cGAMP	Cyclic guanosine adenosine monophosphate
cGAS synthase	Cyclic guanosine monophosphate adenosine monophosphate
cGMP	Cyclic guanosine monophosphate
CO_2	Carbon dioxide
CPT	Camptothecin
CRISPR	Clustered regularly interspaced short palindromic repeats
CSR	Class switch recombination
DDX19	ATP-dependent RNA helicase

DDX5	DEAD box protein 5/ ATP-dependent RNA helicase
DDX9	ATP-dependent RNA helicase A
DHX9	Nuclear DNA Helicase II and RNA Helicase A
DNA	Deoxyribonucleic acid
DNA2	DNA replication ATP-dependent helicase
DNMT	*de novo* methyltransferase
DSB	Double-strand break
dsDNA	Double-stranded DNA
E. coli	*Escherichia coli*
EME1	Crossover junction endonuclease EME1
ERFS	Early replication fragile site
FA	Fanconi anemia
FANCM	Fanconi anemia Complementation group M
FRA3B	Fragile Site, Aphidicolin Type, Common, Fra(3)(P14.2)
G4	G-quadruplex
GTP	Guanosine 5'-triphosphate
H. pylori	Helicobacter pylori
HP	Helicobacter pylori
HBP	Heptose-1,7-bisphosphate
HDAC	Histone deacetylase complex
Ig	Immunoglobulin
IL-1β	Interleukin-1β
IL-8	Interleukin-8
INF	Type I interferon
IRF3	Interferon regulatory factor 3
IκBa	Inhibitor of kappa B alpha
kb	Kilobase
LDL	Low-density lipoprotein
LIG4	DNA ligase 4
LPS	Lipopolysaccharide
MALT	Mucosa-associated lymphoid tissue
MiDAS	Mitotic DNA synthesis
MMR	Mismatch repair

mRNA	Messenger RNA
MUS81	Crossover junction endonuclease MUS81
NER	Nucleotide excision repair
NF-κB	Nuclear factor kappa B
NH_3	Ammonia/nitrogen trihydrate
NOD1	Nucleotide-binding oligomerization domain-containing protein 1
OMPs	Outer membrane proteins
PAMP	Pathogen-associated molecular pattern
PARP1	Poly(ADP-ribose) polymerase
PCNA	Proliferating cell nuclear antigen
PFGE	Pulse-field gel electrophoresis
pH	Power of hydrogen
PINK1	PTEN-induced kinase 1
POLD3	DNA polymerase delta subunit 3
preRNA	Precursor RNA
PRKN	Ubiquitin E3 ligase Parkin
PRR	Pattern recognition receptors
RAD51	DNA repair protein RAD51 homolog 1
RAD52	RAD52 homolog (S. cerevisiae)
RECQ1	RecQ-type helicase type 1
RECQ5	ATP-dependent DNA helicase Q5, RecQ helicase protein-like 5
RNA	Ribonucleic acid
RNAP	RNA polymerase
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RPA	Replication protein A
SETX	Senataxin
SF2	Splicing factor 2
SMARCAL1	SWI/SNF-related matrix-associated actin-dependent regulator of
chromatin	subfamily A-like protein 1
SSB	Single-strand break
ssDNA	Single-stranded DNA
STING	Stimulator of interferon genes

T4SS	Type IV secretion system
TAK1	Mitogen-activated protein kinase kinase kinase 7
TBK1	TANK binding kinase 1
TC-NER	Transcription-coupled nucleotide excision repair
TDP1	Tyrosyl-DNA phosphodiesterase 1
TFIIS	Stimulator of Polymerase II transcription elongation factor
THO	Transcription elongation complex
TIFA	TRAF-interacting protein with forehead-associated domain
TME	Tumour microenvironment
TNF-α	Tumor necrosis factor alpha
TOP1	DNA Topoisomerase 1
TOP1ccs	TOP1 cleavage complex
TRAF	Tumour necrosis factor receptor-associated factor
TRC	Transcription-replication collision
VacA	Vacuolating cytotoxin A
WRN	Werner syndrome ATP-dependent helicase
XPF	Xeroderma pigmentosum Complementation group F
XPG	Xeroderma pigmentosum Complementation group G
XRCC4	DNA repair endonuclease XPF
ZRANB3	Zinc finger Ran-binding domain-containing protein 3
m	Micrometre

1 ABSTRACT

One of the most common infections of a human organism is an infection of stomach induced by pathogenic bacteria *Helicobacter pylori (H. pylori)*. It is estimated that every second person is infected, with even higher prevalence in developing countries. As a quiet enemy, *H. pylori* can colonise a human stomach for decades without manifestation of infection-associated symptoms. However, chronic infection may cause severe damage to the stomach tissue, subsequently leading to the development of gastric diseases, including gastritis and ulcer disease. *H. pylori* infection is also a driving cause of gastric cancer, with 80% of gastric cancers being associated with chronic infection. *H. pylori* ensures its life-long persistence in a human host organism *via* the action of its virulence factors, which have a pleiotropic effect on multiple systems, mostly acting on the attenuation of a human immune system and the induction of atrophy of stomach tissue. The irreversible changes of stomach epithelium are induced by activation of an innate immune response in *H. pylori*-exposed epithelial cells through the stimulation of ALPK1/TIFA/NF-κB signalling pathway upon a recognition of β-ADP heptose, an intermediate product of bacterial lipopolysaccharide biosynthesis, and consequently leading to the formation of DNA double-strand breaks in host cells. We observed that *H. pylori*-induced DNA damage occurs in a manner dependent on an NF-κB-driven transcription, predominantly in cells undergoing DNA replication. In addition, we showed that DNA double-strand breaks are formed as a result of collisions between replication and transcription machineries driven by the accumulation of genotoxic RNA:DNA hybrids, referred to as R-loops, in the host genome. In conclusion, we showed that *H. pylori*-induced oncogenic transformation of stomach tissue might be initiated *via* the excessive formation of DNA double-strand breaks induced as a consequence of R-loop-mediated replication stress in a manner dependent on ALPK1/TIFA/NF-κB signalling pathway.

Keywords: *Helicobacter pylori*, R-loops, replication stress, DNA damage, gastric cancer

1 ABSTRAKT

Jednou z nejrozšířenějších infekcí lidského organizmu je infekce žaludku způsobená patogenní bakterií *Helicobacter pylori* (*H. pylori*). Předpokládá se, že tímto patogenem je nakažená každá druhá osoba a prevalence nákazy výrazně stoupá v méně rozvinutých zemích. *H. pylori*, jako nenápadný nepřítel, může kolonizovat prostředí žaludku po desítky let bez toho, aby se projevil jakýkoliv příznak onemocnění u infikované osoby. Avšak dlouhodobá infekce může způsobit závažné poškození žaludeční tkáně a následné onemocnění žaludku, jakými jsou gastritida, vředová nemoc nebo rakovina. Až 80 % karcinomů žaludku je spojeno s infekcí *H. pylori*, která je považována za hlavní faktor pro rozvoj tohoto onemocnění. Dlouhodobá přítomnost bakterií v lidském hostiteli je zabezpečena produkcí bakteriálních virulentních faktorů, které svou aktivitou utlumují imunitní systém. Nevratné změny epitelu žaludku jsou vyvolané aktivací imunitní odpovědi infikovaných buněk zprostředkované mimo jiné ALPK1/TIFA/NF-κB signální dráhou. Aktivátorem této signální dráhy je β-ADP-heptóza, meziprodukt biosyntézy bakteriálního lipopolysacharidu. Již dříve bylo ukázáno, že infekce buněk *H. pylori* a aktivace ALPK1/TIFA/NF-κB signální dráhy je spojena se zvýšeným výskytem dvouvláknových zlomů v DNA hostitelských buněk. My jsme pozorovali, že poškození DNA vyvolené *H. pylori* se tvoří v závislosti na transkripci cílových genů NF-κB transkripčního faktoru, a především v aktivně se dělících buňkách, které replikují svoji DNA. Dále jsme ukázali, že dvouvláknové zlomy DNA vznikají jako důsledek kolizí mezi replikačními a transkripčními komplexy, které jsou doprovázeny tvorbou genotoxických RNA:DNA hybridů, takzvaných R-smyček, v genomu hostitele. Na závěr jsme ukázali, že nádorová transformace žaludeční tkáně způsobená *H. pylori* může být spuštěna nadměrnou tvorbou dvouvláknových zlomů DNA, které vznikají jako důsledek replikačního stresu, který je v závislosti na aktivaci ALPK1/TIFA/NF-κB signální dráhy vyvolán akumulací R-smyček.

Klíčová slova: *Helicobacter pylori*, R-smyčky, replikační stres, poškození DNA, rakovina žaludku

2 GENERAL INTRODUCTION

2.1 The human pathogen *Helicobacter pylori*

2.1.1 Discovery of *Helicobacter pylori*

The human body is colonised by massive amounts of microorganisms. It is estimated that the microbial cells are as abundant in the human body as the somatic cells (Sender, Fuchs et al. 2016). The most challenging habitat for the majority of bacteria is a human stomach. It was believed that the human stomach is a sterile organ because no microorganism would be able to survive in such a highly acidic environment. In 1982, two scientists, Robin Warren and Barry Marshall isolated a spiral-shaped, Gram-negative and microaerophilic bacterium from the gastric tissue sections from the patients suffering from gastritis (Marshall and Warren 1984), which was later referred to as *Helicobacter pylori* (*H. pylori*). This observation dramatically changed the concepts of gastric microbiology, because until then, it was believed that the primary cause of ulcer disease and gastritis were stress, spicy food and high stomach acid. Marshall self-infected himself by drinking a broth with cultured *H. pylori* to demonstrate that it is a leading cause of gastritis (Marshall, Armstrong et al. 1985). In 2005, Marshall and Warren were awarded the Nobel prize in Physiology and Medicine for their discovery of the bacterium *Helicobacter pylori* and its role in gastritis and peptic ulcer disease.

2.1.2 The morphological features of *H. pylori* contributing to its pathogenicity

H. pylori is 2.5-5 µm long and 0.5-1 µm wide. It has four to six unipolar flagella, which are essential for the mobility of bacterium (Geis, Leying et al. 1989). The motility and spiral shape of the bacterium are necessary for the successful colonisation in the stomach. These two features are vital for *H. pylori* penetration and diffusion into the viscose layer of stomach epithelium to escape from highly acidic pH and peristaltic movements in the stomach (Everhart 2000, Nejati, Karkhah et al. 2018, Tourani, Habibzadeh et al. 2018). Bacteria can exist in three morphological forms, the viable and cultural spiral form, the viable and non-culturable coccoid form and the nonviable form (Andersen and Rasmussen

2009).
The infectious bacteria have a spiral-shaped body and can be isolated from the human stomach. The coccoid form enables the bacteria to survive outside the host organism, for example, in the contaminated water (Everhart 2000, Nejati, Karkhah et al. 2018, Tourani, Habibzadeh et al. 2018).

2.1.3 The infection by *H. pylori* and the factors contributing to disease development

The infection by *H. pylori* is one of the most common chronic bacterial infection worldwide. It affects more than 50% of the world population. Surprisingly, over 80% of infected individuals remain asymptomatic; only less than 20% of infected individuals manifest symptoms of gastric-associated diseases (Garcia, Salas-Jara et al. 2014, Lina, Alzahrani et al. 2014, Percival and Suleman 2014). *H. pylori* infection has been associated with the development of various diseases of the gastrointestinal tract, such as chronic gastritis, ulcer disease, mucosa-associated lymphoid tissue (MALT) lymphoma, and gastric adenocarcinoma. The infection by *H. pylori* represents a significant risk factor for the development of gastric cancer. The risk of developing a gastric disease is positively related to the bacterial strains as well as genetic background of the host. The environmental factors contribute to the differences in *H. pylori* pathogenicity (Peek, Blaser et al. 1999, de Vries, Haringsma et al. 2007). In 1994, the *H. pylori* was classified as a group I carcinogen by the World Health Organisation (1994).

It has been shown that *H. pylori* co-evolved with humans. Thus, it is well adapted for co-existence in the host for a lifetime. *H. pylori* colonises mucosa layer of the human stomach, and the colonisation is commonly acquired during childhood. The transmission of *H. pylori* is poorly understood. Person-to-person transmission within the family seems to be the predominant mode, particularly from mother to children and between siblings (Khalifa, Sharaf et al. 2010). Lifelong colonisation is predicted to be due to an ability of some *H. pylori* strains to evade the host immune response and to withstand the constantly changing gastric environment (Salaun, Linz et al. 2004).

Even though natural habitat for *H. pylori* is the human stomach, the other possible reservoirs for bacterium have been reported. The environment plays a critical role

in the bacterial transmission; for example, the human faecal-contaminated water reservoirs or persistence of the bacteria in biofilms are plausible sources of bacterial infection (Percival and Suleman 2014). Additionally, the zoonotic transmission by houseflies and domestic animals, as well as, iatrogenic transmission have been reported (Tytgat 1995, Grubel, Huang et al. 1998, Neiger and Simpson 2000, Peters, Schablon et al. 2011, Momtaz, Dabiri et al. 2014, Junqueira, Ratan et al. 2017).

2.1.3.1 *H. pylori* virulence factors

H. pylori has developed mechanisms to survive in a highly acidic environment of the human stomach through possession of flagella and urease, ensuring the motility in a high viscose habitat. *H. pylori* needs to establish persistent colonisation of gastric mucosa, which is accomplished by the action of multiple adhesins and other outer membrane proteins. Finally, *H. pylori* possesses a vast repertoire of virulence genes that encode the effector proteins, which directly or indirectly impair the gastric epithelial cells (Kao, Sheu et al. 2016, Sterbenc, Jarc et al. 2019, Whitmire and Merrell 2019).

2.1.3.1.1 Cytotoxin-associated genes pathogenicity island (c*agPAI*)

The principal category of virulence factors are genes encoding cytotoxins, which are not present in all bacterial strains. It has been shown that highly virulent *H. pylori* strains harbour a cytotoxin-associated genes pathogenicity island (*cagPAI*). It is a 40kb region containing multiple genes that mainly encodes the components of the type IV secretion system (T4SS) that enables the bacterium to have intimate contact with the host cell. Through the T4SS, bacteria translocate CagA protein, the only effector protein encoded by *cagPAI* and one of the highly toxic *H. pylori* virulence factor (Backert and Blaser 2016, Sterbenc, Jarc et al. 2019). During the infection, CagA proteins are located on the plasma membrane of bacteria, where they can be phosphorylated. Biological activity of CagA depends on the type and number of phosphorylated motifs. Following its translocation through T4SS, CagA interacts with various host cell molecules, leading to the dysregulation of homeostasis of gastric epithelial cells, through alterations in signal transduction. Additionally, CagA can act directly in an unphosphorylated state and affect cellular tight junctions, polarity, proliferation and inducing a strong inflammatory response.

CagA was pronounced the first bacterial oncoprotein due to its cancer-inducing actions (Jones, Whitmire et al. 2010, Sterbenc, Jarc et al. 2019). The presence of cagA is associated with a higher risk of gastrointestinal diseases and gastric cancer (Sterbenc, Jarc et al. 2019).

2.1.3.1.2Vacuolating cytotoxin A (VacA)

The second prominent *H. pylori* cytotoxin is vacuolating cytotoxin A (VacA). The primary effect of the toxin is an induction of the formation of the vacuoles in eukaryotic cells (Foegeding, Caston et al. 2016, McClain, Beckett et al. 2017, Sterbenc, Jarc et al. 2019). Many *in vitro* studies reported the necessity of toxin activation by acid to increase its vacuolating activity and possibly enhancing its binding to the host cell surface (Khulusi, Ahmed et al. 1995). In contrast to CagA toxin, which needs to be transported through T4SS, the exact mechanism of transport of VacA toxin into the host cell is poorly characterised. It was reported that VacA has pore-forming properties in lipid bilayers *in vitro*. However, another model showed that VacA molecule might be transported into the host cell by receptor-mediated endocytosis.

Multiple activities of VacA toxin has been reported. Apart from the most extensively studied induction of vacuolisation (Atherton, Cao et al. 1995, Foegeding, Caston et al. 2016, Zhang, Xie et al. 2016), VacA also induces a wide range of mitochondrial alterations, including a reduction of mitochondrial transmembrane potential, followed by a release of cytochrome c, activation of Bax, Bak and mitochondrial fragmentation. Exposure of cells to VacA can potentially result in cell death, most probably due to mitochondrial alterations (Yamasaki, Wada et al. 2006, Foegeding, Caston et al. 2016). The effect of VacA toxin is not restricted only to gastric epithelial cells. However, it affects the function of many types of immune cells, including macrophages and lymphocytes (122, 84 in F et al 2016). VacA inhibits activation and proliferation of T- and B- cells and interferes with the antigen presentation in B-cells (Boncristiano, Paccani et al. 2003, Gebert, Fischer et al. 2003, Sundrud, Torres et al. 2004, Foegeding, Caston et al. 2016). All *H. pylori* strains carry *vacA* gene but differ in the vacuolating ability of vacA toxin (Ferreira, Machado et al. 2012). The clinical studies reported an association between various vacA types, especially the more pathogenic, with a higher level of inflammation in gastric mucosa and an

increased risk of gastric atrophy and gastric cancer (Van Doorn, Figueiredo et al. 1999, Sterbenc, Jarc et al. 2019).

2.1.3.1.3 Peptidoglycans

In addition to CagA cytotoxin, the T4SS can also deliver peptidoglycans into the cytoplasm of host cells. Host intracellular pattern recognition protein Nod1 acts as a sensor of peptidoglycan derived from all Gram-negative bacteria. It has been shown that *H. pylori* peptidoglycans activate multiple signalling pathways leading to decreased apoptosis and increased cells migration (Viala, Chaput et al. 2004).

2.1.3.1.4 Lipopolysaccharides

H. pylori, similarly to the other Gram-negative bacteria, expresses several lipopolysaccharides (LPS) on its outer membrane that mediate the adhesion of bacteria to the surface of gastric cells, thus allow persistent colonisation (Hug, Couturier et al. 2010). LPS is composed of three parts: lipid A embedded in the outer membrane, the core oligosaccharide, and the O antigen (Raetz and Whitfield 2002). The ultimate region of the LPS contributing to the virulence of the bacterial pathogen is the O antigen. *H. pylori* mimics carbohydrate structure present on the human epithelial cells or blood cells by incorporating ABO histo-blood group antigens and Lewis antigens on its O chains (Simoons-Smit, Appelmelk et al. 1996). *H. pylori* profits from this mimicry, as the Lewis antigens interact with the human dendritic cells signalling to the immune system to down-regulate host's innate and adaptive immune responses and facilitate immune escape (Bergman, Engering et al. 2004, Hug, Couturier et al. 2010).

2.1.3.1.5 Outer membrane proteins

H. pylori has a massive repertoire of the outer membrane proteins (OMPs), which are present in all bacterial strains. They are responsible for durable colonisation of bacteria through the interactions with host cell surface receptors. *H. pylori* genome encodes more than 30 OMPs. Blood group antigen-binding adhesion protein (BabA) is the best-characterised adhesin on the outer bacterial membrane that enables binding of bacterium to

the ABO histo-blood group antigens (including Lewis [b] antigen) (Ilver, Arnqvist et al. 1998, Roesler, Rabelo-Goncalves et al. 2014). Several clinical studies evaluated the association of the presence of babA with the clinical outcome, and they reported that *babA* gene significantly increases the risk of gastrointestinal diseases, such as ulceration or gastric cancer (Roesler, Rabelo-Goncalves et al. 2014).

2.1.3.1.6 Urease

In order to survive in a highly acidic environment of the stomach, *H. pylori* produces an enzyme called urease, which hydrolyses urea into NH_3 and CO_2. Many studies have reported that urease-defective bacteria are not able to colonise the gastric environment, indicating that urease plays an essential role in bacterial colonisation (Megraud, Neman-Simha et al. 1992, Montecucco and Rappuoli 2001, Roesler, Rabelo-Goncalves et al. 2014). Furthermore, urease activity facilitates motility of bacteria through the mucous layer, which covers the interior of the stomach and acts as a physical barrier against bacterial colonisation. At low pH, gastric mucins form a gel-like structure that effectively traps the bacteria. However, production of ammonium by urease activity raises the pH to be neutral, and the gel-like mucous layer becomes less viscose through which *H. pylori* can swim effortlessly (Celli, Turner et al. 2007, Salama, Hartung et al. 2013).

2.1.3.2 Host factors contributing to the pathogenicity of *H. pylori*

H. pylori specific virulence factors are not absolute determinants of pathogenicity, as a majority of infected individuals remain asymptomatic. It has urged the need to identify the host factors that may influence the *H. pylori*-induced immune response (El-Omar 2001, Wroblewski, Peek et al. 2010). The best-studied host factor affecting the outcome of *H. pylori* infection is cytokine IL-1β. It is a pleiotropic pro-inflammatory molecule that is increased within the gastric mucosa in *H. pylori*-infected patients (Noach, Bosma et al. 1994, Wroblewski, Peek et al. 2010). The multiple clinical studies reported that *H. pylori*-colonised people with high IL-1β production are more susceptible to develop gastric atrophy and gastric adenocarcinoma (El-Omar, Carrington et al. 2000, Wroblewski, Peek et al. 2010).

In addition to IL-1β, TNF-α is a pro-inflammatory acid-suppressive cytokine, that is increased within *H. pylori*-infected gastric mucus. Increased TNF-α expression is associated with an increased risk of gastric cancer (Crabtree, Shallcross et al. 1991, El-Omar, Rabkin et al. 2003, Wroblewski, Peek et al. 2010).

2.1.4 Clinical outcomes of chronic infection by *H. pylori*

2.1.4.1 Gastric diseases

H. pylori may cause direct or indirect damage to the stomach tissue. The disintegration of gastric mucosa due to an activity of virulence factors and induction of apoptosis of epithelial cells in the stomach represents direct damage to the stomach. The indirect damage is due to the induction of chronic immune response (Kobayashi, Lee et al. 2009, Chmiela and Gonciarz 2017).

One of the most frequent gastric diseases strongly correlating with *H. pylori* colonisation is gastritis. Majority of patients infected with *H. pylori* develop a mild or severe form of gastritis, which can be successfully treated by an antibiotic cocktail. Whereas the prognosis of patients with gastritis, not being infected with *H. pylori*, is poor (Kobayashi, Lee et al. 2009, Hagymasi and Tulassay 2014, de Brito, da Silva et al. 2019).

Another example of *H. pylori*-associated gastric disease is peptic ulceration, which occurs in about 10 % of infected individuals. Ulcers form mostly in the two parts of the stomach. Majority of *H. pylori*-induced ulcers are duodenal ulcers. Patients with duodenal ulcers always benefit from antibiotic treatment. Another type is gastric ulcers which form at the isthmus of the stomach. It has been reported that a leading cause of ulcer formation is *H. pylori*-induced production of platelet-activating factor, which acts on angiogenesis (Hagymasi and Tulassay 2014).

Gastric mucosa-associated lymphoid tissue (MALT) lymphoma is a neoplasm (abnormal and excessive growth of tissue) associated with *H. pylori* infection. It is a type of low-grade B-cell lymphomas with tissue infiltration by small lymphocytes. Normal gastric mucosa lacks lymphoid aggregates, only rarely lymphocytes and plasma cells are identified in the gastric mucosa and submucosa. However, *H. pylori* infection triggers an inflammatory response with prominent neutrophil infiltration and lymphocytic

proliferation. The lymphoid cells form lymphoid follicles, which is a characteristic feature for *H. pylori*-associated chronic gastritis. At early stages, gastric MALT lymphoma can be cured by antibiotic-based eradication therapy (Moller, Heseltine et al. 1995, Stolte, Bayerdorffer et al. 2002, Hao, Li et al. 2004, Wroblewski, Peek et al. 2010, Hu, Zhang et al. 2016).

Almost 1 million cases of gastric cancer are diagnosed each year, establishing gastric cancer as the fourth most common cancer and the second leading cause of cancer-related deaths worldwide (Parkin, Bray et al. 2005, Wroblewski, Peek et al. 2010). *H. pylori* causes approximately 80 % of all gastric cancer. Eradication of *H. pylori* significantly decreases the risk of gastric cancer in infected individuals (Wong, Lam et al. 2004, Mera, Fontham et al. 2005, Wroblewski, Peek et al. 2010). Histologically, two distinct variants of gastric cancer have been identified. The first variant is a diffuse-type gastric cancer, which consists of individually infiltrating neoplastic cells, and the second variant is intestinal-type adenocarcinoma (Correa 1996, Wroblewski, Peek et al. 2010).

2.1.4.2 Extra-gastric diseases

Numerous studies reported that *H. pylori* infection is associated with various extra-gastric diseases, including Parkinson's disease, obesity, diabetes mellitus and cardiovascular disease, etc (Franceschi, Covino et al. 2019).

The association between *H. pylori* and cardiovascular diseases has been established based on the studies showing that *H. pylori* may stimulate platelet-activating factor (PAF) and other factors, thus acting on angiogenesis. *H. pylori* may also stimulate atherosclerosis, through the change of lipid profile by increasing the LDL levels, as could be seen in many other infections (Kucukazman, Yeniova et al. 2015, Franceschi, Covino et al. 2019).

Obesity is becoming a global problem, and it was shown that *H. pylori* might affect the prevalence of obesity by persistent damage of gastric mucosa, thereby affecting the level of ghrelin hormone. Ghrelin is produced by endocrine cells in gastric mucosa and is essential for stimulating food intake and feeling of satiety. H. pylori-induced damage to

gastric mucosa reduces the ghrelin concentration, thereby reducing the feeling of satiety, which in turn can lead to obesity (Osawa, Nakazato et al. 2005).

Another example of diseases which can be affected by H. pylori infection is neurodegenerative diseases, such as Parkinson's disease. It has been reported that *H. pylori* is associated with more rapid development of cognitive and functional deterioration. *H. pylori* initiates the destruction of mitochondria, thereby stimulates Parkinson's disease (Kountouras, Boziki et al. 2009, Wong, Rayner-Hartley et al. 2014).

2.1.5 Molecular mechanism of innate immune response induction by *H. pylori*

Screening of *H. pylori* transposon library showed that genetic ablation of *cagPAI*-encoded T4SS hinders the stimulation of innate immune response, suggesting that T4SS is a critical bacterial factor contributing to H. pylori-induced immune response. Except for the bacterial factors, the critical components of innate immune signalling in host cells needed to be identified. It was reported that T4SS-dependent delivery of bacterial factors stimulates the innate signalling leading to the activation of a global transcription factor nuclear factor kappa B (NF-κB) and the expression of multiple pro-inflammatory genes, including cytokines, such as interleukin-8 (IL-8). Prior to this observation, most of the IL-8 response in gastric epithelial cells had been attributed to NOD1 activation by peptidoglycans, which subsequently leads to the NF-κB activation. Later on, the tumour necrosis factor receptor-associated factor (TRAF)-interacting protein with forehead-associated domain (TIFA) has been reported as a critical host cell component of the innate signalling pathway, downstream of *cag*-T4SS-induced signalling (Gall, Gaudet et al. 2017).

Inductor of TIFA activation is a bacterial metabolite heptose-1,7-bisphosphate (HBP) present in the cytosol of host cell. HBP is a highly conserved metabolite among all Gram-negative bacteria, which is generated through ADP-L-glycero-β-D-manno-heptose (ADP-heptose) biosynthesis pathway in the bacterial cytosol (Kneidinger, Marolda et al. 2002, Gall, Gaudet et al. 2017, Pfannkuch, Hurwitz et al. 2019). The final product of biosynthesis, ADP-heptose, is then incorporated into the core part of *H. pylori* LPSs (Gaudet, Sintsova et al. 2015, Gaudet and Gray-Owen 2016, Gall, Gaudet et al. 2017) (Figure 1). The LPS molecule is assembled in the bacterial cytosol, transported across the periplasm, flipped out, and become a part of the outer bacterial membrane. LPS and many

other OMPs are part of the pathogen-associated molecular pattern (PAMP), which is a characteristic mark of microbial pathogens. Recognition of PAMPs by pattern recognition receptors (PRRs) on the host cell membrane represents the front line of cell self-defence against microbial infection (Medzhitov 2007, Pfannkuch, Hurwitz et al. 2019). Based on the knowledge of the infection by other Gram-negative pathogenic bacteria, it was suggested that HBP is delivered to the host cell cytosol by endocytosis of extracellularly released HBP (observed in *Neiserria* or *Salmonella* infection) or through intracellular bacterial replication (in case of *Shigella flexneri* infection). However, in case of *H. pylori* infection, the HBP is presented to TIFA signalling pathway by its T4SS-dependent translocation, which is a new type of HBP delivery mechanism among the Gram-negative bacteria (Gaudet, Sintsova et al. 2015, Gaudet and Gray-Owen 2016, Gall, Gaudet et al. 2017).

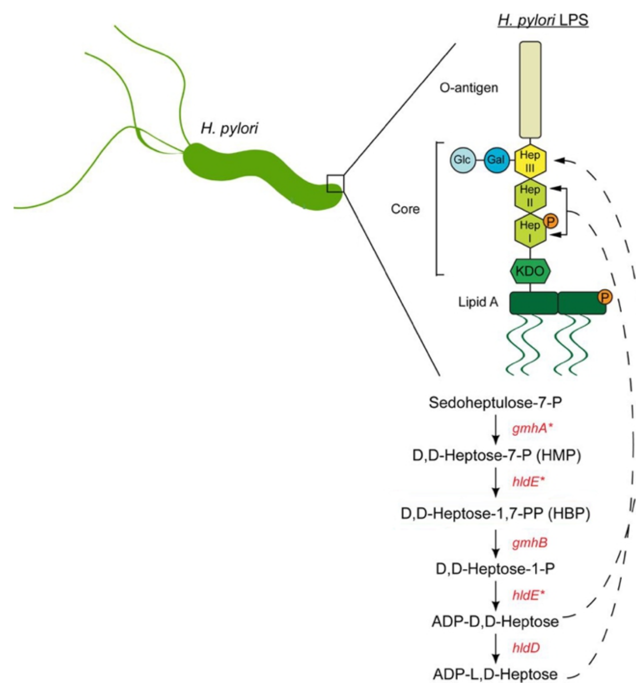

Figure 1: The biosynthesis of LPS. In the first step, sedoheptulose-7-P is converted into D-α,β-D-heptose 7-P by isomerase GmhA. In the second step, a bifunctional enzyme with kinase and adenyltransferase activity, HldE, phosphorylates the primary product to D-*glycero*-D-*manno*-heptose 1,7-bisphosphate (HBP), which is later dephosphorylated by phosphatase GmhB to D-β-D-heptose 1-phosphate. In the fourth step, ADP-D-β-D-heptose is activated by HldE, followed by an epimerisation to ADP-L-b-D-heptose (ADP heptose) by epimerase HldD (adapted from (Gall, Gaudet et al. 2017).

Upon *H. pylori* infection, the immune response is initiated by cytosolic delivery of HBP that is sensed by ALPK1 kinase, which then phosphorylates TIFA proteins (Gall, Gaudet et al. 2017, Milivojevic, Dangeard et al. 2017). Phosphorylated TIFA forms oligomers that act as a scaffold for recruitment of multiple proteins involved in NF-κB signalling (TIFAsomes), including TRAF6. TIFAsomes activate (NF-κB). The NF-κB family proteins, including p65/p50, are sequestered in the cytoplasm by an inhibitor of kappa B alpha (IκBa). Upon activation, the IκB kinase (IKK) phosphorylates IκBa, which is subsequently degraded. Inhibitor-unbound p65/p50 heterodimer translocates into the nucleus. The activation of NF-κB leads to the up-regulation of pro-inflammatory genes expression and production of cytokines, such as interleukin-8 (IL-8) (Figure 2) (Hacker and Karin 2006, Hoffmann and Baltimore 2006, Hartung, Gruber et al. 2015, Zimmermann, Pfannkuch et al. 2017, Pfannkuch, Hurwitz et al. 2019).

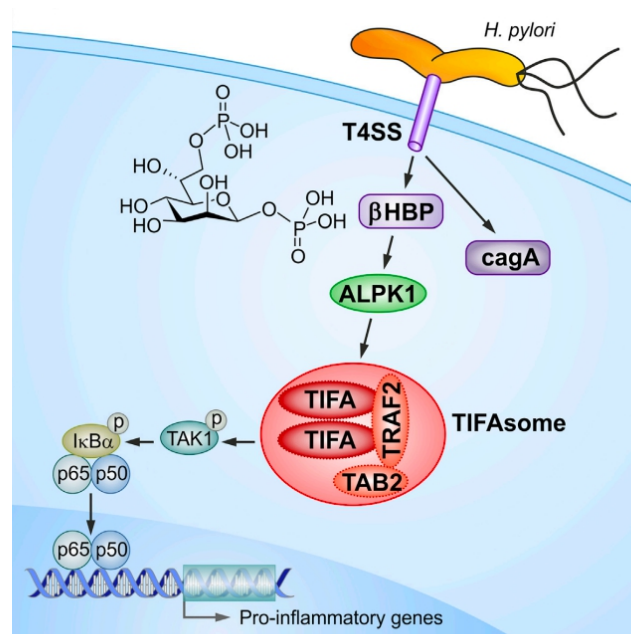

Figure 2: Graphical scheme of innate immune response induction in gastric epithelial cells exposed to *H. pylori* (adapted from (Zimmermann, Pfannkuch et al. 2017).

Following TIFA activation, peptidoglycans delivered through T4SS activate NOD1 leading to the amplification of NF-κB activation. Finally, CagA, the only known effector protein encoded by *cagPAI*, associates with host TAK1, enhances TAK1 polyubiquitination, which is mediated by E3 ligase activity of TRAF6. Polyubiquinated

TAK1 activates NF-κB leading to even further amplification of innate immune response (Gall, Gaudet et al. 2017).

A more recent study showed that HBP is not a TIFA-activating bacterial factor. The amount of HBP in lysates of *H. pylori* is surprisingly low. This level is not sufficient for the induction of such a strong inflammatory response in *H. pylori*-exposed gastric cells. ADP-heptose, the final product of LPS biosynthesis, was identified as a potential inductor of the innate immune response. ADP-heptose is an abundant metabolite in *H. pylori* lysate, and it is a potent PAMP recognised by ALPK1/TIFA signalling pathway (Pfannkuch, Hurwitz et al. 2019). Surprisingly, incubation of adenocarcinoma-derived gastric cells (AGS) with a synthetically-prepared analogue of ADP-heptose led to a robust NF-kB response, while HBP stimulated the immune response only when it was delivered to the cytoplasm by transfection reagent (Gall, Gaudet et al. 2017, Zimmermann, Pfannkuch et al. 2017, Pfannkuch, Hurwitz et al. 2019).

2.1.6 Genomic instability associated with *H. pylori* infection

In addition to the pathological effects of the *H. pylori*-specific infection on the gastric mucosa, several studies provided evidence that *H. pylori* promotes carcinogenesis of gastric epithelial cells by threatening the integrity and stability of the host cell's genome (Machado, Figueiredo et al. 2010, Toller, Neelsen et al. 2011). The observation that human pathogenic bacteria jeopardise genome by activating multiple signalling pathways leading to DNA double-strand breaks (DSBs) is not limited only to *H. pylori*. Multiple species of pathogenic bacteria have been reported to damage the nuclear DNA (Nougayrede, Homburg et al. 2006, Toller, Neelsen et al. 2011, Hartung, Gruber et al. 2015). This was first reported for genotoxins-expressing strains of *Escherichia coli,* which induce DNA DSBs, trigger mammalian DNA damage repair processes, ultimately leading to cell cycle arrest and cell death (Nougayrede, Homburg et al. 2006). It has been demonstrated that exposure of gastric epithelial cells to *H. pylori* induces fragmentation of DNA detected by pulsed-field gel electrophoresis (PFGE), suggesting that *H. pylori* exhibits genotoxic activities (Toller, Neelsen et al. 2011). However, the molecular mechanism of DSB formation induced by *H. pylori* is unknown. Interestingly, a recent study has shown that DSBs induced by *H. pylori* infection were reduced by depletion of the nucleotide excision

repair (NER) endonucleases, XPG and XPF, or by inhibition of transcription (Hartung, Gruber et al. 2015). Given the earlier observation of the requirement of XPG and XPF for R-loop-dependent DNA breakage (Sollier, Stork et al. 2014), a possibility exists that *H. pylori*-induced DSBs result from accumulation of co-transcriptional R-loops. Results presented in this thesis strongly support this hypothesis.

2.2 Co-transcriptional RNA:DNA hybrids (R-loops)

2.2.1 Formation of co-transcriptional R-loops

RNA:DNA hybrids are essential intermediates in many fundamental cellular processes, including the replication of lagging DNA strand, regulation of gene expression and maintaining the stability of telomeres (Westover, Bushnell et al. 2004, Aguilera and Garcia-Muse 2012, Niehrs and Luke 2020). While term RNA:DNA hybrids refers to base pairing of RNA with DNA, the term "R-loop" describes three-stranded structures formed by the RNA:DNA hybrid and a displaced single-strand DNA (ssDNA) (Figure 3). The most accepted model of R-loops formation, commonly known as a "thread-back" model, suggests that during the ongoing transcription, the free end of newly synthesised RNA invades the underwound DNA duplex behind the transcription complex to pair with the template DNA strand, while displacing the non-template strand as a ssDNA loop (Figure 4C) (Westover, Bushnell et al. 2004). The majority of R-loops are formed co-transcriptionally during the ongoing transcription. However, a small percentage of R-loops could also be formed in *trans*, when RNA is produced at a spatially distinct site (Wahba, Gore et al. 2013, Niehrs and Luke 2020).

Figure 3: Schematic representation of R-loop structure (adapted from (Hamperl and Cimprich 2014)

The molecular mechanism of R-loop formation has been elucidated mostly from *in vitro* transcription experiments utilizing synthetic RNA:DNA hybrid structures. The factors contributing to the formation of R-loops *in vivo* and factors enhancing the stability of RNA:DNA hybrids remain mostly unknown. R-loops are formed in a sequence-independent manner. However, the critical factor for their formation is high G-content in the non-template strand, whereas the high G-content within RNA:DNA hybrid structure, which is also required for the elongation of R-loops (Figure 4A) (Roy and Lieber 2009,

Aguilera and Garcia-Muse 2012). Other factors on non-template DNA strand may drive the R-loops formation and enhance the hybrid stability, including the DNA secondary structures and nicks on the non-template DNA strand, which reduce the ability for displaced non-template DNA strand to re-anneal with template strand (Figure 4B) (Roy, Zhang et al. 2010, Hamperl and Cimprich 2014).

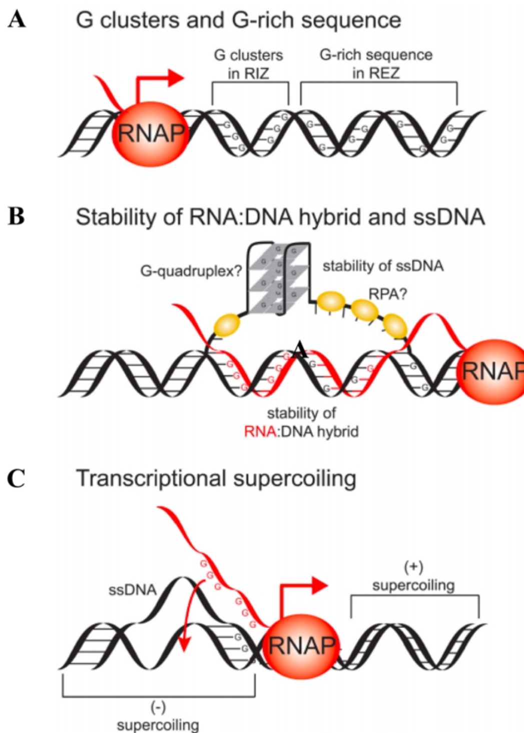

Figure 4: Formation of R-loops. (A) R-loop formation and elongation depend on a G-content. The G-rich clusters in the initiation zone (RIZ) help to initiate the formation of R-loop. G-rich sequences in the elongation zone (REZ) are necessary for elongation of R-loop. (B) Stability of R-loop is enhanced by forming DNA secondary structures, such as G4, on a displaced ssDNA. (C) Thread-back model explaining the formation of co-transcriptional R-loops (adapted from (Hamperl and Cimprich 2014).

2.2.2 Factors preventing the formation of R-loops

Numerous reports showed that genome instability is caused predominantly by problems in DNA replication. However, another critical cellular process strongly affecting the stability of the genome is DNA transcription (Huertas and Aguilera 2003, Li, Wang et al. 2005). In budding yeast, the mutants of THO complex, which is a four-protein complex involved in transcription elongation, exhibited an increased level of R-loops. THO-defective yeast cells also manifested an elevated level of DNA breaks, an essential prerequisite

for chromosomal rearrangements, which were induced in R-loops-dependent manner (Huertas and Aguilera 2003, Wahba, Amon et al. 2011). Additionally, the chromosomal rearrangements were observed more frequently in yeast with defective transcription regulation, such as *sin3*Δ yeast lacking the master repressor of transcription (Wahba, Gore et al. 2013). It suggests that excessive transcription compromises the genome integrity, most probably through the accumulation of R-loops (Tous and Aguilera 2007, Chan, Hieter et al. 2014, Wahba, Costantino et al. 2016).

Since the original observation in yeast mutants of THO complex, the many RNA processing and export factors have been implicated in the prevention of R-loop formation (Huertas and Aguilera 2003, Li, Wang et al. 2005). It was suggested that eukaryotic cells prevent the hybrid formation by coating the nascent RNA with proteins involved in preRNA processing and export, therefore restricting the association between nascent RNA and the template DNA strand (Liu and Wang 1987, Manis, Tian et al. 2002, Luna, Gaillard et al. 2008, Luna, Rondon et al. 2012, Masani, Han et al. 2013, Hamperl and Cimprich 2014). Presumably, the protective role against R-loop formation is a feature of only a specific group of RNA-binding proteins, especially those being involved in the assembly of mRNA-protein particles during transcription elongation (Aguilera and Garcia-Muse 2012) (Figure 5). Even though the accumulation of incorrectly processed preRNA in the nucleus and a high level of transcription increase the chances of RNA:DNA hybrids formation, they are not sufficient for the massive accumulation of R-loops, which are a prerequisite for induction of genome instability (Tous and Aguilera 2007, Garcia-Rubio, Chavez et al. 2008, Garcia-Muse and Aguilera 2019). In the vertebrate cells, R-loops-mediated genome instability was first documented in a chicken B cell line depleted of the splicing factor ASF1/SF2 (alternative splicing factor 1). Inactivation of ASF1 resulted in DSBs formation and chromosomal rearrangements (Li, Wang et al. 2005, Hamperl and Cimprich 2014). The expression of R-loop-resolving factor, RNase H1, suppressed the chromosomal DNA fragmentation, suggesting that formation of R-loops has the potential to be a source of DNA damage in the absence of proper splicing of mRNA (Hamperl and Cimprich 2014).

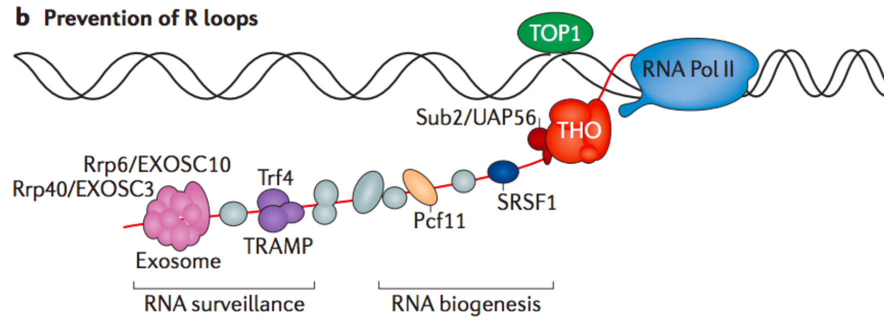

Figure 5: Prevention of R-loops formation by coating a nascent RNA with mRNA-processing proteins in metazoans (Adapted from (Santos-Pereira and Aguilera 2015).

Another critical factor constraining the ability of the nascent RNA to hybridise with DNA is the topology of DNA. The negative supercoiling, which is transiently generated behind the active RNA polymerase, is favouring R-loop formation because it makes the double-strand DNA prone to be open. In eukaryotic cells, DNA topoisomerase 1 (TOP1) can relax the DNA supercoiling forming as a consequence of ongoing transcription (Drolet, Phoenix et al. 1995, El Hage, French et al. 2010). The compromised activity of TOP1, for example by the TOP1 inhibitor camptothecin, is linked with an elevated level of co-transcriptionally formed R-loops (Drolet, Phoenix et al. 1995, Tuduri, Crabbe et al. 2009, El Hage, French et al. 2010, Garcia-Muse and Aguilera 2019).

Chromatin conformation is an additional factor protecting the genome from the accumulation of R-loops. The role of chromatin in the regulation of R-loop formation was first observed in budding yeast, in which the non-lethal mutation of histone H3 and H4 increased the level of R-loops (Garcia-Pichardo, Canas et al. 2017). In human cells, mapping of genomic regions prone to form R-loops (R-loops-positive regions) strongly correlates with the open-chromatin regions, rather than regions with condensed chromatin (Chan, Hieter et al. 2014, Sanz, Hartono et al. 2016, Garcia-Muse and Aguilera 2019). The increased R-loops accumulation was also reported in SIN3A histone deacetylase complex (HDAC)-deficient cells. In the absence of histone deacetylase activity, the chromatin is in a relaxed state, which has been associated with active gene expression (Johnstone 2002, Iizuka and Smith 2003, Ropero and Esteller 2007). Additionally, SIN3A histone deacetylase interacts with the RNA processing/export THO complex, suggesting that THO complex prevents the R-loops

by ensuring a proper nascent RNA processing and by promoting local histone deacetylation, thus ensuring the closed chromatin conformation (Herrera-Moyano, Mergui et al. 2014, Salas-Armenteros, Perez-Calero et al. 2017).

2.2.3 Factors resolving R-loops

Despite all the mechanisms that cells have developed to prevent the formation of R-loops, they may fail, and R-loops can be formed. Therefore, cells have evolved backup mechanisms to resolve R-loops and to avoid their unscheduled accumulation in the genome. The most studied R-loops-resolving factors are the proteins belonging to the RNase H family. Theses enzymes can specifically cleave the RNA strand within RNA:DNA hybrid. The substrates for RNase H could arise from numerous cellular processes, such as synthesis of RNA primers during the replication of a lagging strand, the formation of R-loops during transcription, reverse transcription, misincorporation of ribonucleotides by DNA polymerases (Cerritelli and Crouch 2009). The most eukaryotic organisms have at least one of two classes of RNase H enzymes. Mammalian cells have RNase H1 and RNase H2 (Figure 6A) (Nowotny, Gaidamakov et al. 2007, Cerritelli and Crouch 2009, Hyjek, Figiel et al. 2019).

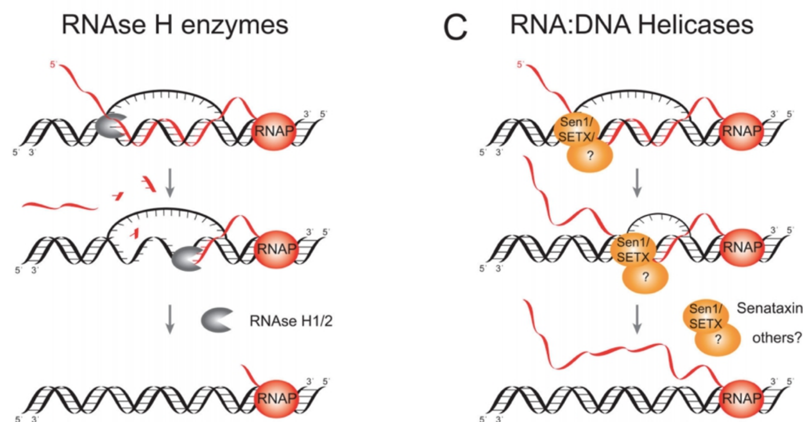

Figure 6: Resolution of R-loops. (A) RNase H1/ RNase H2 degrades an RNA moiety within RNA:DNA hybrid. (B) RNA-binding helicases, such as Senataxin, unwind the RNA:DNA hybrid and allow re-annealing of the DNA strands (adapted from (Hamperl and Cimprich 2014).

RNase H1 is the endonuclease specific for RNA:DNA hybrids with a minimal length of four base pairs. The human RNase H1 interacts with 11 base pairs of the RNA:DNA hybrids (Nowotny, Gaidamakov et al. 2007). The enzyme consists of an N-terminal hybrid-binding domain linked by a connection domain to a C-terminal catalytic domain (Cerritelli and Crouch 2009). In most of the eukaryotic organisms, RNase H1 can be localized into mitochondria and nucleus. RNase H1 knock out mice die at early stages of embryonic development due to a failure in the replication of the mitochondrial genome, suggesting an essential role of RNase H1 in a generating or removing of RNA primer during mitochondrial DNA replication (Cerritelli, Frolova et al. 2003). The nuclear isoform of RNase H1 plays an essential role in the elimination of RNA:DNA hybrids generated during replication, thereby preventing genome instability associated with the formation of R-loops (Huertas and Aguilera 2003, Cerritelli and Crouch 2009). The ectopic expression of RNase H1 has been shown to suppress phenotypes arising from different R-loop-promoting stress conditions, including the absence of a proper mRNA processing or the absence of topoisomerase activity (Drolet, Phoenix et al. 1995, Tous and Aguilera 2007, Garcia-Rubio, Chavez et al. 2008, El Hage, French et al. 2010, Aguilera and Garcia-Muse 2012). RNase H1 is presumably recruited to hybrids *via* a direct interaction with replication protein A (RPA), an ssDNA-binding protein coating the displaced DNA strand of R-loop. RPA directly enhances the association of RNase H1 with RNA:DNA hybrid *in vitro* (Bhatia, Barroso et al. 2014, Nguyen, Yadav et al. 2017). RPA binding-defective RNase H1 fails to associate with R-loops and suppress the R-loop-associated genome instability. RPA is known to be a sensor of R-loops (Nguyen, Yadav et al. 2017).

RNase H2 is a trimeric enzyme, consisting of three subunits (A, B and C), with RNase H2A possessing a nuclease activity. RNase H2 recognises and removes the RNA from RNA:DNA hybrids and initiates an excision of ribonucleotides mistakenly incorporated into the DNA, a process known as a ribonucleotide excision repair (RER) (Eder, Walder et al. 1993, Cerritelli and Crouch 2009, Reijns and Jackson 2014, Bartsch, Knittler et al. 2017). Additionally, RNase H2 is involved in the removal of RNA primers in Okazaki fragments via the interaction with a proliferating cell nuclear antigen (PCNA), a protein responsible for recruiting DNA polymerase and other factors involved in the processing of Okazaki fragments to the replication fork (Murante, Henricksen et al. 1998,

Cerritelli and Crouch 2009). RNase H2 knock out mice show early embryonic lethality due to increased incorporation of single ribonucleotides into genomic DNA resulting in extensive DNA damage (Hiller, Achleitner et al. 2012, Reijns, Rabe et al. 2012, Bartsch, Knittler et al. 2017). Mutations in RNase H2 have been associated with the development of Aicardi-Goutières syndrome (AGs), a severe neurological disorder frequently addressed as a Mendelian mimic of congenital viral infection of the brain. It is an autoimmune-like disorder characterised by microcephaly, basal ganglia calcification and elevated levels of lymphocytes and interferon alpha in the cerebrospinal fluid (Cerritelli, Frolova et al. 2003, Reijns, Rabe et al. 2012, Reijns and Jackson 2014). It was reported that AGs-associated RNase H2 mutations impair the excision repair leading to the elevated level of DNA damage arisen from the misincorporated ribonucleotides, which in turn leads to the activation of innate immune sensing pathways and the production of many pro-inflammatory genes, such as interferons (Brzostek-Racine, Gordon et al. 2011, Rigby, Webb et al. 2014, Hartlova, Erttmann et al. 2015, Bartsch, Knittler et al. 2017, Shapson-Coe, Valeiras et al. 2019).

RNase H2 was described as a "house-keeping" enzyme removing a majority of RNA:DNA hybrids, including misincorporated ribonucleotides and R-loops in a post-replicative manner, while RNase H1 is a rather a stress responder, as it resolves replication stress-associated R-loops (Lockhart, Pires et al. 2019). Overexpression of wild-type RNase H1 is widely used to revert phenotype attributed to R-loops formation and such rescue is considered as an indirect evidence for R-loops formation (Drolet, Phoenix et al. 1995, Hamperl and Cimprich 2014). We and others have also used RNase H1 (only DNA binding domain or catalytically inactive enzyme) for detection and isolation of R-loops (Aguilera and Garcia-Muse 2012, Teloni, Michelena et al. 2019).

An increasing number of RNA-dependent ATPases with RNA:DNA hybrid unwinding activity have been identified as a potential R-loops-resolving factors, namely human Senataxin (Figure 6B), Aquarius, DHX9, DDX19, DDX5, BLM, and many others (Skourti-Stathaki, Proudfoot et al. 2011, Cristini, Groh et al. 2018, Garcia-Muse and Aguilera 2019). Depletion of these proteins leads to the accumulation of R-loops, suggesting their involvement in R-loops-removing mechanisms. Senataxin (SETX) is a helicase involved in the removal of R-loops formed at transcription termination sites.

Additionally, SETX was shown to localize to the sites of the replication-transcription collisions (Steinmetz, Warren et al. 2006, Hamperl and Cimprich 2014). The mutations in *SETX* are linked to the neurodegenerative disorders, such as ataxia with oculomotor apart 2 and amyotrophic lateral sclerosis type 4 (Sun, Yabuki et al. 2001, Strasser, Masuda et al. 2002, Hamperl and Cimprich 2014). Similarly to Senataxin, depletion of the putative RNA/DNA helicase Aquarius leads to the accumulation of R-loops and R-loops-dependent DNA damage (Sollier, Stork et al. 2014). In case of other RNA-dependent helicases, such as Pif1, BLM, DDX9 and FANCM, the RNA:DNA unwinding activity was reported *in vitro*. It has not been determined whether these helicases are able to unwind RNA:DNA hybrids *in vivo.* Recent studies identified several DEAD-box helicases, such as DDX19, in the proteomic analysis of factors associated with the metabolism of RNA:DNA hybrids *in vivo* (Cristini, Groh et al. 2018, Wang, Grunseich et al. 2018). DDX19 is a nucleopore-associated mRNA export factor, which is potentially able to unwind RNA:DNA hybrids (Hodroj, Recolin et al. 2017). Further validations are required to conclude the implication of identified helicases in the removal of R-loops.

2.2.4 RNA:DNA hybrids with a physiological/regulatory function

From bacteria to human cells, RNA:DNA hybrids are structures with a two-faced nature. They are essential intermediate products in cellular processes, for example, in lagging-strand synthesis during replication, *E. coli* plasmid replication, mitochondrial genome replication, transcription termination regulation and immunoglobulin (Ig) class switch recombination in mammalian B cells. Besides these roles, R-loop may be a harmful structure, jeopardizing the genome integrity and stability (Figure 7) (Reaban and Griffin 1990, Pavri 2017).

One of the best-studied examples of naturally-occurring R-loops with regulatory function is the Immunoglobulin class switch recombination (CSR) process generating antibody diversity. In mammals, CSR occurs by intrachromosomal deletional recombination between the Ig switch (S) regions in order to change the antibody class. Upon transcription, the transcripts of S region are prone to form R-loops (Yu, Chedin et al. 2003). In response of antigen presentation to mature B-cells, the R-loops within the Ig S regions are targeted by B-cell specific cytidine deaminase, called activation-induced

deaminase (AID) (Muramatsu, Sankaranand et al. 1999, Muramatsu, Kinoshita et al. 2000, Revy, Muto et al. 2000, Roy, Yu et al. 2008, Stavnezer, Guikema et al. 2008). AID deaminates cytosines to uracils in a displaced ssDNA within R-loops, which is the first critical step for generating DNA DSBs responsible for CSR (Petersen-Mahrt, Harris et al. 2002, Bransteitter, Pham et al. 2003, Yu, Huang et al. 2004, Roy, Yu et al. 2008). Following AID action, uracil glycosylase (UNG) excises the deaminated C generating an abasic site, which is subsequently cut by apurinic/apyrimidinic endonuclease (APE1) generating a single-strand break (SSB) (Poltoratsky, Goodman et al. 2000, Petersen-Mahrt, Harris et al. 2002, Stavnezer, Guikema et al. 2008). DSBs might arise from SSBs that are close to each other and are localised on opposite DNA strands or by mismatch repair process (Stavnezer and Schrader 2006, Stavnezer, Guikema et al. 2008). Finally, the DNA breaks can lead to aberrant recombination necessary for CSR (Figure 7E) (Imai, Slupphaug et al. 2003, Rada, Di Noia et al. 2004, Fan, Matsumoto et al. 2006, Roy, Yu et al. 2008, Stavnezer, Guikema et al. 2008).

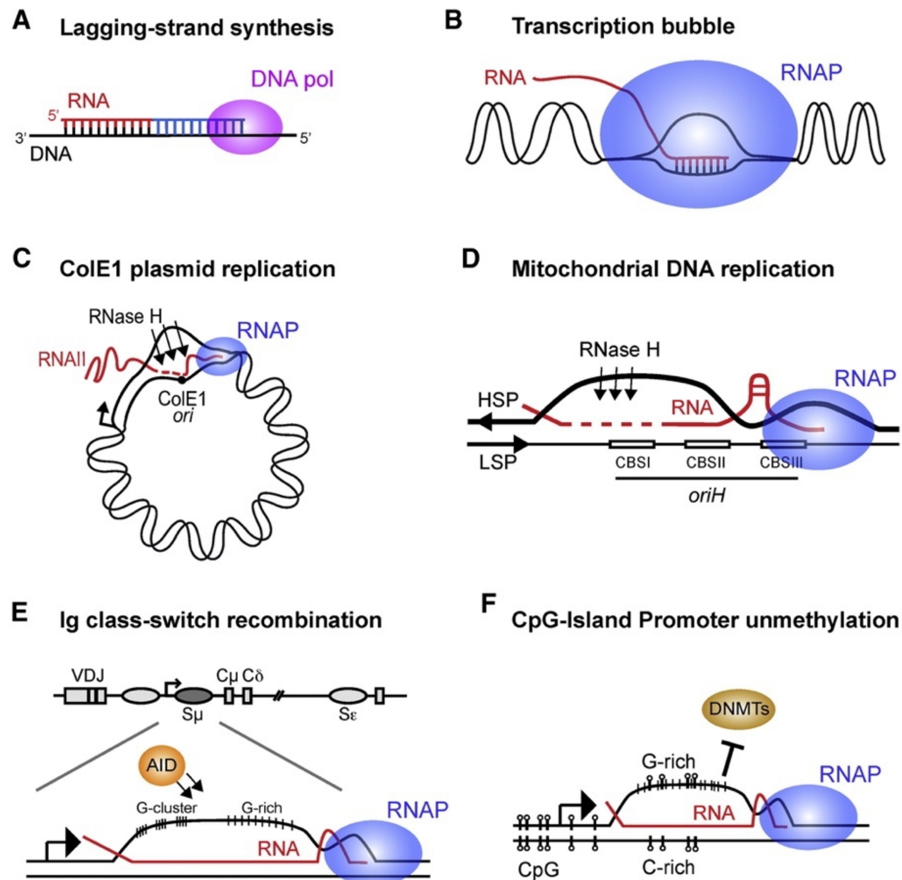

Figure 7: Physiological role of RNA:DNA hybrids and R-loops. (A) RNA:DNA hybrids form during the synthesis of lagging strand in eukaryotic DNA replication. (B) RNA:DNA hybrids within the transcription bubble. (C) R-loops in bacterial plasmid replication. (D) R-loops in mammalian mitochondrial DNA replication. (E) R-loops in Ig class switch recombination. (F) R-loops formation protects CpG island promoters from *de novo* DNA methyltransferases (DNMTs) (adapted from (Aguilera and Garcia-Muse 2012).

2.2.4 Co-transcriptionally formed R-loops as a source of genome instability

Aberrant processing or failure to resolve R-loops gives rise to DSBs. Moreover, the involvement of R-loops in the induction of DSBs during the Ig CSR highlights the potential for co-transcriptional R-loops to induce DNA breakage and consequently, threaten the stability of the genome.

2.2.4.1 The single-stranded DNA breaks as a source of R-loop-mediated genome instability

The excessive formation of R-loops may increase the amount of ssDNA in the genome by displacing the non-template DNA strand, which is more susceptible to DNA-damaging agents than a DNA duplex. The candidate factor damaging displaced ssDNA is AID, a DNA-specific cytidine deaminase involved in DSBs formation during Ig CSR (Drolet, Phoenix et al. 1995, Chaudhuri, Khuong et al. 2004, Debatisse, Le Tallec et al. 2012, Hamperl and Cimprich 2014, Debatisse and Rosselli 2019). Alternatively, the transiently displayed ssDNA within R-loop may fold into a secondary structure, referred to as G quadruplexes, which are recognised by G4-specific endonucleases that cleave within the single-stranded regions of the individual G quartets (Nickoloff 1992, Ursic, Himmel et al. 1997, Hamperl and Cimprich 2014). In addition to the vulnerability of displaced ssDNA, other structural features of R-loops contribute to making these structures a target for nucleases. The junctions connecting the RNA:DNA hybrid and ssDNA are recognised by specific endonucleases, referred to as flap-endonucleases, which may be another source for DNA breakage within the R-loop structure (Wang 2002, Darzacq, Shav-Tal et al. 2007, Hamperl and Cimprich 2014).

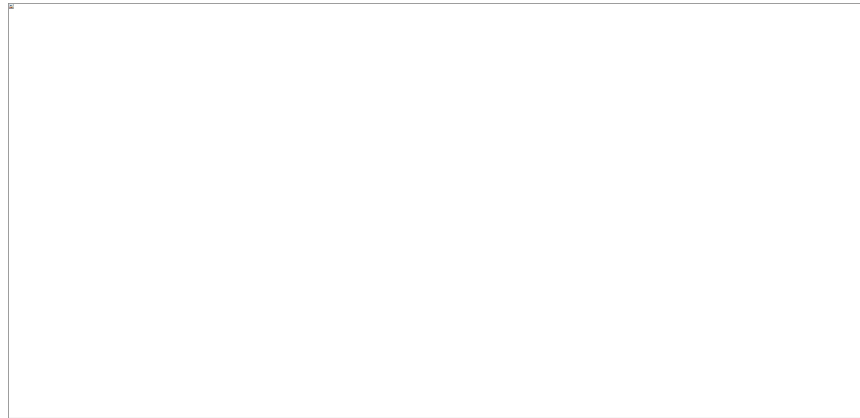

Figure 8: Mechanisms for R-loop-mediated formation of singe-strand breaks (SSBs). (A) SSB can be generated by AID activity followed by MMR or BER repair. (B) G-quadruplex (G4)-specific endonucleases may recognize a secondary DNA structure on the ssDNA and generate an SSB (adapted from (Hamperl and Cimprich 2014)).

There are multiple plausible mechanisms generating single-strand breaks within the R-loop structure. However, the threat with more detrimental effect on genome integrity resulting from R-loop accumulation are DSBs. The mechanisms implicated in DSBs formation as a consequence of R-loops accumulation has not been elucidated completely. One possible mechanism is derived from CSR, where two proximal SSBs localized on the opposite strands are processed in the way resulting in the DSB formation (Wellinger, Prado et al. 2006, Hamperl and Cimprich 2014). It can be speculated that AID-mediated deamination, base-excision repair process, and the activity of different endonucleases may work together to generate DSB and to induce the chromosomal fragility. In addition, R-loops may be recognized and processed by TC-NER endonucleases XPG and XPF (Figure 10C). Such processing may lead directly to DSB formation or the formation of SSBs, that can ultimately cause fork collapse during replication (Sollier, Stork et al. 2014).

2.2.4.2 R-loop-mediated conflicts between replisome and transcription complexes

Numerous recent findings suggest that DNA damage induced by the accumulation of co-transcriptional R-loops occurs due to R-loop-mediated impairment of DNA replication. R-loop formation may cause RNA polymerase pausing, which interferes with the progression of replication fork due to collision of the replication fork and the transcription machinery.

It was first observed in the yeast THO mutants, where R-loop-mediated chromosomal fragility was documented only in sequences transcribed in S-phase (Westover, Bushnell et al. 2004, Hamperl and Cimprich 2014). It was also observed in yeast that replication forks pause more frequently in highly transcribed genes (Wongsurawat, Jenjaroenpun et al. 2012, Hamperl and Cimprich 2014). Moreover, replication fork slowing in TOP1-deficient human cells can be rescued by the ectopic expression of RNase H1. All these observations are suggesting that DNA breakage may arise from R-loop-mediated transcription-replication collisions (TRCs), consequently leading to a slowing or stalling of replication forks, which is commonly referred to as replication stress (Rada, Di Noia et al. 2004, Hamperl and Cimprich 2014).

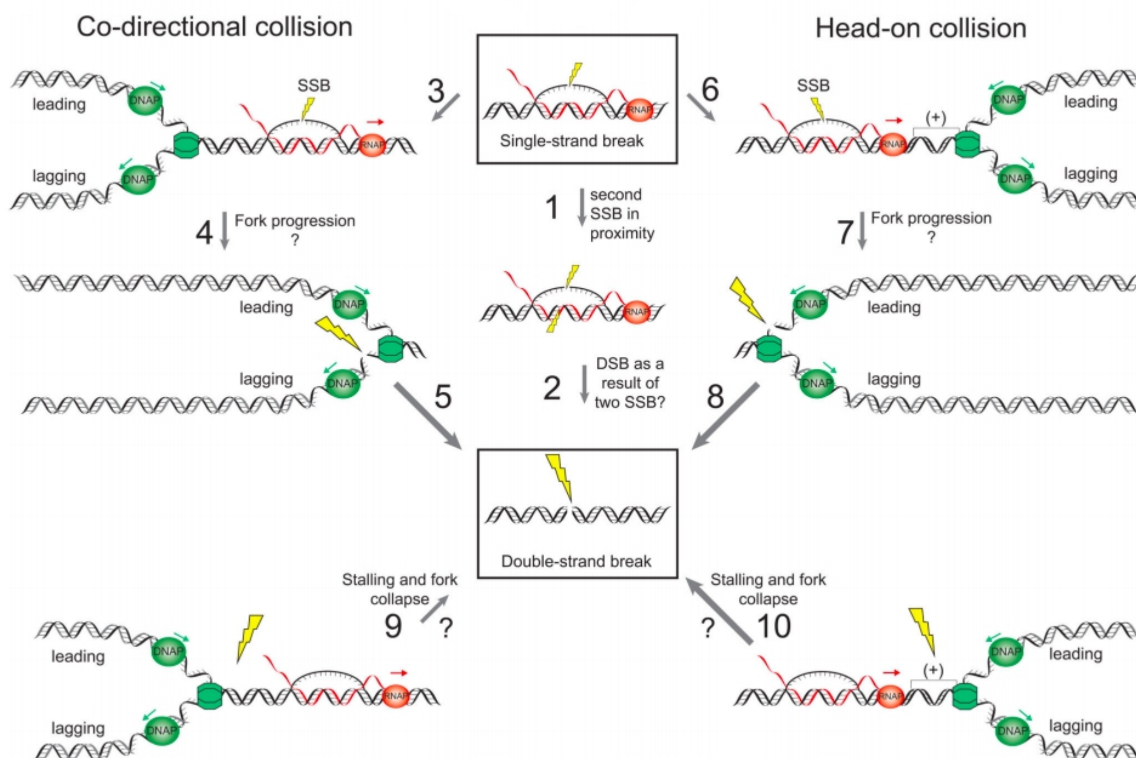

Figure 9: R-loops mediated double-strand breaks formation. Replication fork encounters the R-loops containing SSB in co-directional (3) or in head-on collision (6). Fork progression over the hybrid (4, 7) would result in a DNA lesion in lagging strand (5) or leading strand (8). In the absence of initiating lesion, the stalled RNA polymerase may collide with replisome in a co-directional (9) or head-on (10) orientation, leading to a fork stalling or collapse, consequently to a DSB (adapted from (Hamperl and Cimprich 2014)

Transcription and replication are two essential processes for cell viability and proliferation. However, when the machineries mediating these processes encounter each other, the replication fork cannot progress past the elongating RNA polymerase, leading to a conflict (Azvolinsky, Giresi et al. 2009, Merrikh, Machon et al. 2011, Garcia-Muse and Aguilera 2019). The collisions between replisome and transcription machinery represent a significant source of genomic instability. There are two types of collisions: a) co-directional collision, DNA and RNA polymerases move in the same direction; b) head-on collision, polymerase progress from the opposite direction towards each other (Figure 9). There are multiple ways how R-loop can be associated with TRCs. The involvement of R-loops in blocking of replication fork progression, thereby increasing a chance for conflicts to occur, can be an indirect consequence of SSB formation in ssDNA within R-loops. This initiating lesion ensures the separation of the parental strand and progression of replication fork through the break would result in DSB on the lagging strand in case of co-directional collision (Figure 9:3-5) or on the leading strand in case of head-on collision (Figure 9:6-8) (Hamperl and Cimprich 2014). Alternatively, the R-loops may directly impede the replisome progression and lead to DSBs without the requirement of an initiating lesion (Figure 9:9-10). Additionally, during elongation, both RNA and DNA polymerases change the structure of chromatin, which may hinder the progression of polymerases. The convergence of RNA polymerase and replication fork, when oriented head-on, leads to an accumulation of a positive DNA supercoiling between polymerases, consequently resulting in fork stalling, and inhibition of both processes (Bermejo, Lai et al. 2012).

The continually emerging evidence suggest that progression of replisome is impaired predominantly by head-on collisions with more detrimental effects on genome integrity. However, the co-directional collisions between replisome and R-loop structure may also contribute to genome instability (Baaklini, Usongo et al. 2008, Hamperl and Cimprich 2014). The RNA strand of R-loop may provoke an aberrant DNA replication by providing a free 3’ end to the stalled replication machinery (Hamperl and Cimprich 2014).

2.2.4.3 Transcription-replication conflicts as a source of replication stress and DNA damage at specific genomic loci

There are regions in the human genome, referred to as common fragile sites (CFSs), that are hotspots for chromosomal instability and are frequently associated with rearrangements in cancers (Aguilera and Garcia-Muse 2012, Aguilera and Garcia-Muse 2013, Hamperl and Cimprich 2014). CFSs contain difficult-to-replicate DNA sequences prone to form secondary structures, such as hairpins, which may act as a physical barrier for replication fork progression, leading to replication fork stalling or collapse. Late replication timing is a frequently observed feature of CFSs. For example, the replication of FRA3B gene occurs in very late S-phase even under unperturbed condition (Glover, Berger et al. 1984, Li and Wu 2020). Additionally, CFSs often contain very large genes (> than 800 kb), which extend their transcription into the S phase of a subsequent cell cycle. This would increase a chance of replication-transcription encounters (Helmrich, Ballarino et al. 2011). At the sites of concurrent transcription and replication, the functional replication fork would help to resolve the collision between replication and transcription complexes. However, in the presence of replication stress, the impaired replication fork will block the transcription elongation complex, thus leading to the increased R-loop formation at the sites of paused RNA polymerase (RNAP) complex (Wu, Shyy et al. 1988, Helmrich, Ballarino et al. 2011, Hamperl and Cimprich 2014, Li and Wu 2020). Such collisions result in CFS expression, the term commonly referring to CFS breakage on metaphase chromosomes (Glover 2006, Durkin and Glover 2007, Li and Wu 2020). Interestingly, overexpression of RNase H1 prevents the chromosome breakage at the long CFS-associated genes induced by mild replication stress. In contrast, siRNA-mediated RNase H1 knock down led to an increase of breakage at CFS regions. It suggest that R-loop formation at the long genes participate in the high rate of DNA breakage at CFS regions (Helmrich, Ballarino et al. 2011). The maintenance of CFS stability is highly prioritized in order to prevent detrimental chromosomal breakage during replication. Translesion DNA synthesis, a mechanism to replicate through the structure-forming DNA sequences at CFSs, and the activity of DNA helicases, including BLM and WRN, and translocases, such as Fanconi anaemia (FA) proteins, are two major mechanisms to resolve DNA secondary structures when forks are

stalled at CFSs to maintain CFS stability (Howlett, Taniguchi et al. 2005, Pirzio, Pichierri et al. 2008, Wang, Grunseich et al. 2018, Debatisse and Rosselli 2019, Li and Wu 2020).

Interestingly, the cells have one last chance to finish replication of under-replicated DNA of difficult-to-replicate loci, such as CFSs, to prevent the chromosome breakage and potentially lethal chromosome missegregation during mitosis. This unconventional DNA synthesis, termed as mitotic DNA synthesis (MiDAS), occurs in early mitotic prophase. It was shown that under the condition of replication stress, entry of the cells into mitotic prophase triggers the recruitment of MUS81 endonuclease to CFSs, which then promotes POLD3-dependent DNA synthesis at CFSs to minimise the chromosome nondisjunction during the cell division (Minocherhomji, Ying et al. 2015). Additionally, RECQ5 was shown to be essential for MiDAS. RECQ5 disrupts the RAD51 nucleoprotein filament, which is formed on stalled replication forks at CFSs to protect the fork from nucleolytic degradation. RECQ5 *via* the direct interaction with MUS81, is recruited to CFSs during the early mitosis and facilitates MUS81/EME1-mediated cleavage, thus triggers MiDAS (Di Marco, Hasanova et al. 2017). RAD51 together with BRCA1, are known suppressor of MiDAS (Schlacher, Christ et al. 2011). We have shown that MiDAS depends on the R-loops (Chappidi, Nascakova et al. 2020), thereby it is possible that a fraction of chromosome missegregation observed in MiDAS-deficient cells might arise from the failure of restart of R-loop-stalled replication forks in early mitosis.

During the cell division, the broken chromosomes could not be incorporated into the daughter nuclei and they form small-sized nuclei, referred to as a micronuclei (Fenech, Knasmueller et al. 2016). They represent a cytological marker of chromosome mis-segregation. It has been shown that frequency of micronuclei formation is increased upon the depletion of MUS81 and RECQ5, indicating that MiDAS in required to counteract the potentially fatal chromosome missegregation (Naim, Wilhelm et al. 2013, Di Marco, Hasanova et al. 2017).

Genome-wide mapping of localization of repair proteins identified a new type of DNA regions vulnerable to breakage upon replication stress. These genomic loci are commonly known as early replication fragile sites (ERFS). CFSs replicate late with breaks manifested on metaphase chromosomes, while ERFSs replicate early with breaks appeared mainly in S or G2 phase the cell cycle. ERFSs are characterized by their close proximity to

replication origins, colocalization with highly expressed genes and enrichment for repetitive motifs. Similarly to CFSs, ERFSs are prone to form DNA secondary structures when DNA is in single-stranded conformation, which potentially stalls DNA replication. In contrast to CFSs, ERFSs present high level of transcription, which suggest that replication stress might be linked to R-loops, and consequently result in DSBs formation (Barlow, Faryabi et al. 2013, Sarni and Kerem 2016, Li and Wu 2020).

2.2.4.4 Mechanisms to suppress TRCs

The significant consequence of transcription-replication collisions is genome instability triggered by chromosome breakage that results as a consequence of replication fork blocking or collapse. The continual progression of replisome can be impaired by elongating, paused or backtracked RNAP complex, which acts as a roadblock for replication fork. Thus, cells have evolved several mechanisms to resolve the transcriptional blocks and to regulate the rate of transcription to limit transcription-associated genome instability. Reactivation of a stalled RNAP complex may be mediated by the subunit of RNAP complex, referred to as TFIIS (the stimulator of Pol II transcription elongation factor), which stimulates the cleavage of a transcript to restart arrested RNA polymerase (Cheung and Cramer 2011, Hamperl and Cimprich 2016). Moreover, it possesses an RNA-proofreading activity to maintain transcriptional fidelity (Thomas, Platas et al. 1998). Additionally, the human RECQ5 helicase decreases the elongation rate of transcription to ensure the continual progression of the RNAP complex (Figure 10, left part) (Saponaro, Kantidakis et al. 2014, Hamperl and Cimprich 2016).

The progression of RNA polymerase can also be impaired by lesions in the DNA template and by the formation of transcription-associated RNA:DNA hybrids. The transcription can proceed only if the lesions are repaired by transcription-coupled nucleotide excision repair pathway (TC-NER) (Figure 10, middle part). The resolving of RNAP complex stalling due to formation of R-loops is mediated by RNA:DNA helicases, including SETX or AQR or by RNase H enzymes.

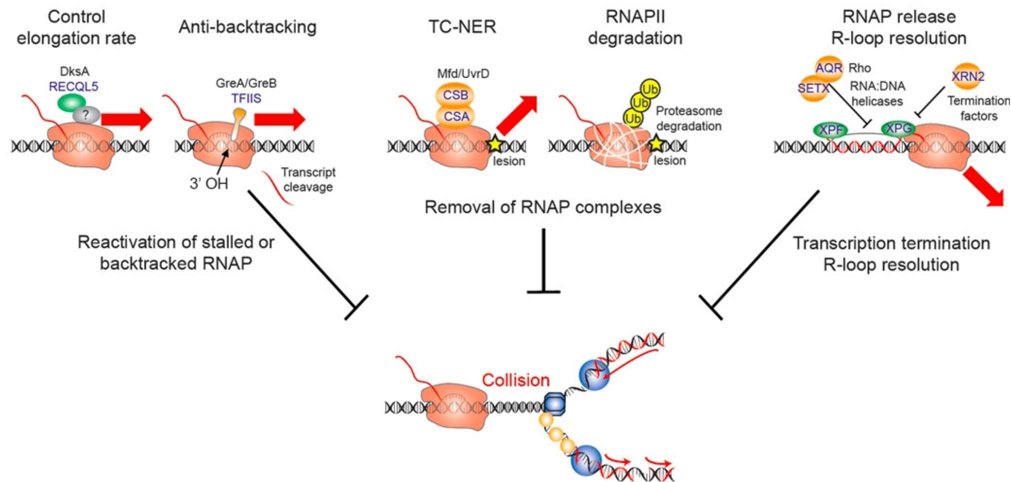

Figure 10: Transcription-associated mechanisms to suppress TRCs. (left) Reactivation of a stalled or backtracked RNAP complex mediated by the activity of TFIIS or RECQ5. (middle) Removal of lesion by TC-NER. If TC-NER fails, the arrested RNAP complex is degraded by proteasome. (right) Transcription termination and resolution of R-loops is mediated by XNR2 exonuclease and RNA/DNA helicase, including SETX, AQR. R-loops may be recognized and processed by NER endonucleases XPG and XPF (adapted from (Hamperl and Cimprich 2016).

Replication fork stalled at transcription complexes can resume DNA synthesis by different DNA repair and fork restart pathways. A stalled replication fork can be rescued by the firing of an adjacent dormant origin (Figure 11: i). Alternatively, restart of replication fork stalled at sites of R-loop-induced TRCs may require the activity of additional factors involved in DNA recombination (Prado and Aguilera 2005). TRC-induced stalled replication forks are stabilized by ATR, BRCA2 and Fanconi anaemia DNA repair pathway and restarted by BRCA2-dependent recombination (Roy, Chun et al. 2011, Garcia-Rubio, Perez-Calero et al. 2015) (Figure 11: ii). Additionally, BRCA1 was shown to suppress R-loops formation and processing via its interaction with SETX (Roy, Chun et al. 2011). A prolonged fork stalling may also promote re-annealing of the parental strands priming for fork reversal. This could stabilize the fork structure and provide the time necessary for the resolution of the transcriptional block. Removal of the block can then promote fork restart (Figure 11: iii). However, if the transcription block persists, restart could be initiated by break formation and recombination-mediated processes to overcome the obstacle (Figure 11: iv) (Gomez-Gonzalez, Felipe-Abrio et al. 2009).

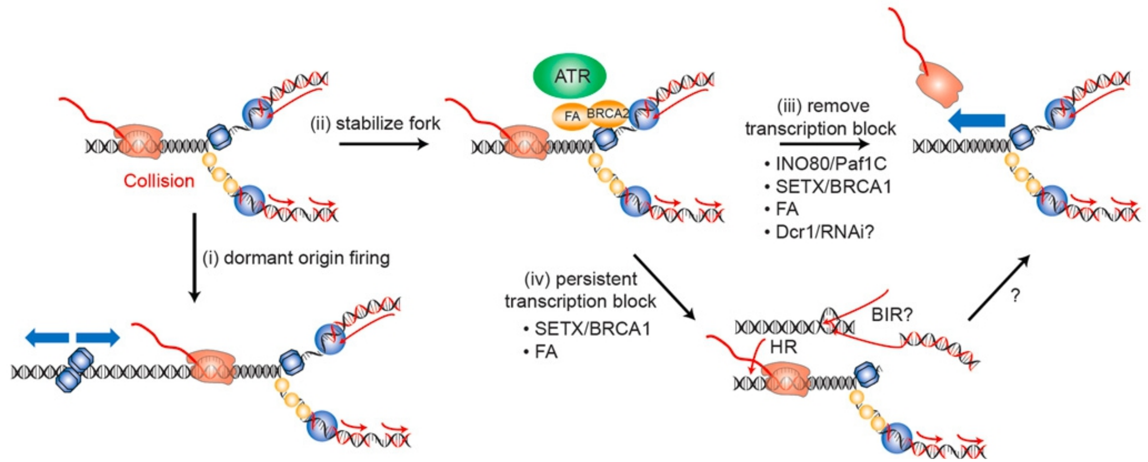

Figure 11: Replication-associated mechanisms to prevent and resolve TRCs. (i) Stalled replication fork can resume DNA synthesis by firing of an adjacent dormant origin. (ii) Replication fork s stalled at TRCs may be stabilized by ATR, BRCA2 and FA pathway. (iii) Prolonged stalling of replication fork may promote fork reversal. Removal of transcription block by RNA:DNA hybrid resolving factors promotes the replication fork restart. (iv) Persistent transcription block ultimately leads to fork breakage. Recombination-dependent repair mechanisms are used to overcome the obstacle (adapted from (Hamperl and Cimprich 2016)

Under the replication stress, paused replication fork at the CFSs in early prophase of mitosis requires for its restart fully functional MiDAS to prevent deleterious consequences of under-replicated chromosomes present during the cell division. This thesis addresses a question whether the same set of proteins is required for the restart of R-loops-stalled replication fork in S phase of the cell cycle. We postulated a model demonstrating the mechanism of resolution of transcription-replication collision to ensure the restart of DNA replication and RNA synthesis and to avoid potentially hazardous consequences of fork reversal.

3 AIMS OF THE STUDY

The presented PhD thesis is focused on how genotoxic stress contributes to the oncogenic transformation of human cells. The main goal of the thesis is to elucidate the molecular mechanisms contributing to the genotoxic stress induced by *Helicobacter pylori* infection.

The specific aims of the thesis are:

- to evaluate the role of the ADP-heptose/ALPK1/TIFA/NF-κB signalling axis in the induction of DNA damage in *H. pylori*-exposed gastric epithelial cells
- to explore the role of genotoxic RNA:DNA hybrids (R-loops) and R-loop-dependent replication stress in *H. pylori*-induced DNA damage

These aims I have addressed as shared first author in the Research paper #1.

Along with the main goal, this thesis addresses several other questions covered in Research paper #2 and #3, to which I contributed as a co-author:

- to characterise the molecular mechanism underlying the restart of replication forks blocked by co-transcriptional R-loops
- to unravel the mechanism of cancer cell escape from human immune surveillance system

4 PRESENTED PUBLICATIONS AND AUTHOR CONTRIBUTION

Research paper #1

ALPK1/TIFA/NF- B axis links a bacterial carcinogen to R-loop-induced replication stress

Bauer, M.*, Nascakova, Z.*, Mihai, A. I.*, Cheng, P. F., Levesque, M. P., Lampart, S., Hurwitz, R., Pfannkuch, L., Dobrovolna, J., Jacobs, M., Bartfeld, S., Dohlman, A., Shen, X., Gall, A. A., Salama, N. R., Topfer, A., Weber, A., Meyer, T. F., Janscak, P., Muller, A. (*shared first-authorship)

Nat Commun. 2020 Oct 9;11(1):5117. doi: 10.1038/s41467-020-18857-z

Zuzana Nascakova, as a shared first-author, contributed to the publication by elaborating the experiments using the techniques of immunofluorescence microscopy. She performed the acquisition of all immunofluorescence images of cells using widefield or confocal microscopes and performed the high-throughput analysis of acquired data. She created work pipelines for automated immunofluorescence image analysis using specialised software, such as Fiji, ScanR Analysis and CellProfiler. She contributed actively to the preparation of the manuscript.

Research paper #2

Fork Cleavage-Religation Cycle and Active Transcription Mediate Replication Restart after Fork Stalling at Co-transcriptional R-Loops

Chappidi, N., Nascakova, Z. Boleslavska, B. Zellweger, R. Isik, E., Andrs, M., Menon, S., Dobrovolna, J., Balbo Pogliano, C., Matos, J., Porro, A., Lopes, M. Janscak, P.

Mol Cell. 2020 Feb 6;77(3):528-541.e8. doi: 10.1016/j.molcel.2019.10.026

Zuzana Nascakova, as a co-author, contributed to the paper by performing a part of the immunofluorescence microscopy experiments. She optimised the protocol for R-loop

detection by staining with an antibody recognising RNA:DNA hybrids (S9.6) and created a work pipeline for automated software-based analysis of immunofluorescence signal.

Research paper #3

Cancer-Cell-Intrinsic cGAS Expression Mediates Tumor Immunogenicity
Schadt, L., Sparano, C., Schweiger, N. A., Silina, K., Cecconi, V., Lucchiari, G., Yagita, H., Guggisberg, E., Saba, S., Nascakova, Z., Barchet, W., van den Broek, M.
Cell Rep. 2019 Oct 29;29(5):1236-1248.e7. doi: 10.1016/j.celrep.2019.09.065

Zuzana Nascakova, as a co-author, contributed to the paper by performing a part of the experiments requested for the re-submission of revised manuscript. She performed the microscopic analysis of DNA damage markers upon the irradiation of the cell lines harbouring studied mutations.

5 COMMENTS ON PRESENTED PUBLICATIONS

5.1 *Helicobacter pylori* promotes carcinogenesis *via* induction of the R-loop-driven replication stress

The innate immune system represents the front line of defence against invading microbial pathogens and therefore plays a crucial role in the early recognition of pathogen and triggering the pro-inflammatory response. The critical part of innate immune response is the detection of evolutionarily conserved structures on the surface of microbial pathogens, referred to as pathogen-associated molecular patterns (PAMPs) by a protein family of pattern recognition receptors (PRRs). The lipopolysaccharides (LPS) are part of the PAMPs in all bacterial pathogens (Medzhitov and Janeway 2000, Mogensen 2009). It is widely accepted that intermediate products of LPS biosynthesis are known inducers of the innate immune response in host cells upon infection with various Gram-negative bacterial pathogens. The transport of bacterial metabolites to the epithelial cells in the host organism differs across the bacterial pathogens. In the case of *H. pylori* infection, the active inducer of immune response is ADP-heptose, which is transported into the cytosol of gastric epithelial cells through a functional T4SS secretion system (Gall, Gaudet et al. 2017, Stein, Faber et al. 2017, Zimmermann, Pfannkuch et al. 2017). T4SS acts as a molecular syringe for bacteria to inject its metabolite into the cytoplasm of host cells. Upon the delivery of ADP-heptose, the host cell responds to the infection by stimulation of the innate immune response comprised of APLK1/TIFA/NF-B signalling axis. *H. pylori* infection induces DNA damage by introducing a DNA double-strand breaks into a genome of its host cell. However, the nature of these breaks is not known. Whether *H. pylori*-induced DNA breaks depend on T4SS-transported ADP-heptose and the innate immune signalling and how DNA breaks are introduces into the genome of host cells is addressed by this thesis.

5.1.1 NF-κB-driven transcription as a source of *H. pylori*-induced DNA damage

Infection of cells with *H. pylori* leads to the formation of DNA double-strand breaks in the host genome. The innate immune response to infection by most of the Gram-negative bacteria, including *H. pylori*, is comprised of ALPK1/TIFA/NF-κB signalling axis. The stimulation of this pathway leads to the activation of NF-κB, a dominant transcription factors driving the early response to the *H. pylori* infection. The T4SS/ALPK1/TIFA-dependent activation of NF-κB results in the translocation of NF-κB into the nucleus of the host cell and the induction of gene expression of its target genes, such as pro-inflammatory genes (Greten, Eckmann et al. 2004, Hanada, Uchida et al. 2014, Taniguchi and Karin 2018). We have shown that *H. pylori*-induced DNA damage is abolished entirely upon the inhibition of transcription and suppression of NF-κB activation, and depends upon the ALPK1/TIFA branch of the NF-κB activation triggered by ADP-heptose, an LPS biosynthetic intermediate transported into the host cell via the T4SS secretion system (Bauer, Nascakova et al. 2020). NF-κB was previously identified as a critical factor linking the inflammation and carcinogenesis, since the NF-κB-driven genome instability has been linked with various types of cancers, including colon and liver cancer (Greten, Eckmann et al. 2004, Pikarsky, Porat et al. 2004, Taniguchi and Karin 2018). It was shown that NF-κB activation plays a dual role in the tumour microenvironment (TME). In cancer cells, NF-κB induces DNA damage by enhancing the production of reactive oxygen/nitrogen species (ROS/RNS) and by promoting the cell survival *via* the activation of anti-apoptotic genes (Luo, Kamata et al. 2005). The effect of NF-κB is not restricted only to cancer cells, but its activation in the multiple cell types of TME, such as immune cells, promotes the growth of tumour *via* the production of cytokines (such as TNF-α), chemokines and pro-angiogenic factors (Grivennikov, Karin et al. 2009). In addition, our study demonstrated that NF-κB activation threatens the genome stability through the formation of co-transcriptional R-loops, which are frequently associated with replication stress, consequently leading to DNA damage (Bauer, Nascakova et al. 2020).

5.1.2 DNA damage induced by *H. pylori* infection results from R-loop accumulation in the genome of host cell

We have reported that the infection by *H. pylori* leads to the formation of DNA double-strand breaks in a manner dependent on the T4SS/ALPK1/TIFA/NF-κB axis (Bauer, Nascakova et al. 2020). The question arises as to how *H. pylori* induces DNA DSBs? We have shown that DNA damage induced by *H. pylori* infection is formed as a consequence of the R-loops-driven transcription-replication collisions (Bauer, Nascakova et al. 2020). Previously, it was reported that the *H. pylori* virulence factor CagA, the only known cagPAI-encoded effector protein is responsible for genotoxic properties of *H. pylori*, but later, it was shown that the cytotoxicity mediated by CagA has tremendous effects on infected gastric epithelial cells and immune cells, but it does not contribute to the *H. pylori*-induced DNA DSB formation (Hanada, Uchida et al. 2014). The study by Hartung et al. demonstrated that DNA DSBs are introduced by nucleotide excision repair endonucleases, XPG and XPF (Hartung, Gruber et al. 2015). Based on the mechanism of nuclear hormone receptor-regulated transactivation of target genes *via* the XPG-mediated introduction of DNA DSBs in the promoter region, they reported that XPG/XPF-mediated DNA DSBs serve as a prerequisite for the activation of NF-κB target gene expression (Le May, Mota-Fernandes et al. 2010, Le May, Fradin et al. 2012, Hartung, Gruber et al. 2015). Interestingly, these results agree with the model of R-loop-driven DNA DSBs formation. Hartung et al. observed that siRNA-mediated silencing of XPG or XPF reduced the fragmentation of DNA (assessed by PFGE) upon *H. pylori* infection. However, this reduction was partially suggesting that other mechanism might be involved in DSB induction. Additionally, it is widely accepted that R-loops, induced by the absence of RNA processing factor or helicases, such as Senataxin or Aquarius, or by TOP1 inhibition, are processed into DNA DSBs by the XPG/XPF endonucleases (Sollier, Stork et al. 2014). Recently, Cristini et al. (2019) reported that DNA transcription could induce DNA DSBs, also in non-replicating cells (Sordet, Redon et al. 2009, Cristini, Ricci et al. 2019). They demonstrated that DSBs arise from two neighbouring SSBs on opposite DNA strands produced during the transcription. The first SSB arises from the removal of transcription-blocked TOP1 cleavage complex (TOP1ccs). TOP1 relaxes the torsional stress on DNA by

forming a transient TOP1ccs, which could be trapped on chromatin under the pathological condition and act as a roadblock for RNA polymerases. The resolution of transcription-blocked TOP1ccs is dependent on TDP1-mediated excision repair. The second SSB is formed as a result of cleavage of the R-loops by endonucleases, including XPG and XPF (Pommier, Sun et al. 2016, Cristini, Ricci et al. 2019).

We have shown that *H. pylori*-induced DNA DSBs arise from the R-loops-stalled replication forks (Bauer, Nascakova et al. 2020). The level of DNA DSBs, as well as the level of R-loops, was strongly enhanced upon the *H. pylori* infection. Interestingly, the DNA DSBs were suppressed by the ectopic overexpression of RNase H1, an enzyme resolving R-loop structures by degrading the RNA strand within RNA:DNA hybrid, suggesting that DNA damage is indeed caused by R-loops accumulation in the genome of gastric epithelial cells exposed to *H. pylori*. R-loops can be formed as a consequence of head-on collisions of replisome and transcription complex, where they can halt the progression of RNA polymerase, which in turns can lead to blocking of replication fork (Hayden and Ghosh 2014, Garcia-Rubio, Perez-Calero et al. 2015, Lang, Hall et al. 2017). The transcription-replication collisions (TRCs) are frequently observed within the long genes, which need more than one cell cycle to finish their gene expression. In the following S phase of the cell cycle, the RNA polymerase complexes act as roadblocks for ongoing replication forks, leading to the TRCs (Hayden and Ghosh 2014, Garcia-Rubio, Perez-Calero et al. 2015). Regardless, whether *H. pylori*-induced formation of R-loops is a result of head-on TRCs or their accumulation in genome leads to the formation of TRCs, we have shown that *H. pylori* infection negatively affects the progression of replication through the excessive accumulation of R-loops in the genome of host cells in a manner dependent on T4SS/ALPK1/TIFA/NF-κB pathway (Figure 12) (Bauer, Nascakova et al. 2020). Our study links the innate immune response activation to DNA damage and carcinogenesis.

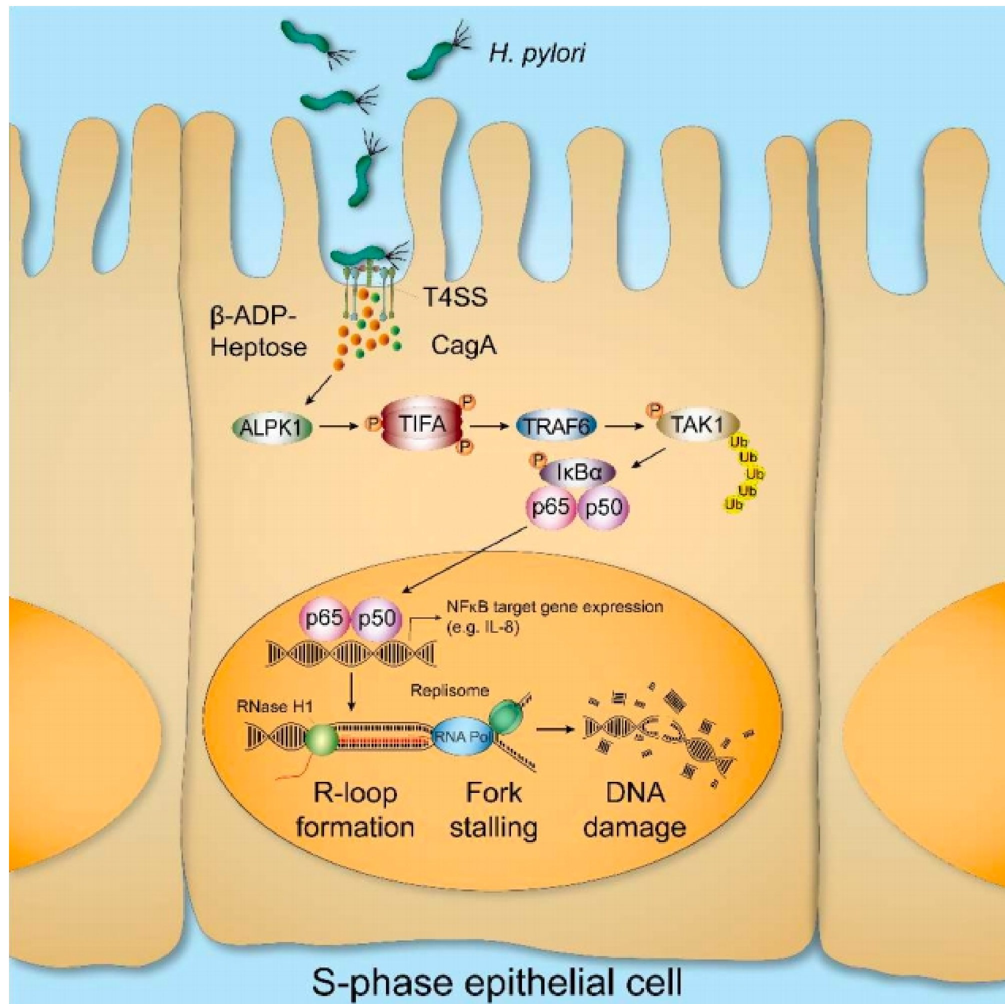

Figure 12: The schematic model of H. pylori- induced R-loop formation and DNA damage (Adapted from Bauer, Nascakova et al. 2020) .

5.2 Resolution of R-loops-mediated transcription-replication collisions

During DNA replication, replisome frequently encounters various obstacles. In the worst scenario, the progression of the replication fork is irreversibly blocked, leaving the parts of the genome under-replicated (Zeman and Cimprich 2014). One of the most prominent obstacles for replisome progression is the transcription elongation complex. The replication fork and RNA polymerase can encounter each other while moving in the same direction (co-directional collision) or in the opposite direction (head-on collision) (Zeman and Cimprich 2014). Transcription-replication collisions (TRCs) in head-on orientation promote the formation of co-transcriptional R-loops that can further block the progression of replication forks (Hamperl and Cimprich 2014, Zeman and Cimprich 2014, Hamperl, Bocek et al. 2017). R-loops formation is facilitated by the negative DNA supercoiling generated behind the ongoing RNA polymerase complex (Duquette, Handa et al. 2004, Zeman and Cimprich 2014). Additionally, converging replication fork and RNA polymerase complex create positive DNA supercoiling in between them, which further enhances the chance of replication fork progression failure. The TRCs are potentially hazardous events, and thereby cells have evolved mechanisms to prevent them, such as possession of the factors resolving R-loops (Hamperl and Cimprich 2014, Zeman and Cimprich 2014, Garcia-Muse and Aguilera 2016). However, the mechanism of DNA synthesis restart following the replication fork blockage by an R-loops remains unexplained.

5.2.1 Fork reversal as a response of cell to the replication stress

Following perturbation of replisome progression, the replication fork is remodelled into a four-way junction, a DNA transaction referred to as a fork reversal. It is an evolutionary conserved and strictly regulated process to ensure replication completion, chromosome integrity and DNA damage response. The fork reversal is initiated by coordinated annealing of the two newly synthesised strands and re-annealing of the parental strands to form a regressed arm at the fork elongation point (Neelsen and Lopes 2015, Santos-Pereira and Aguilera 2015). The fork reversal limits genome instability by maintaining the

forks in a stable paused conformation, allowing more time for DNA damage repair or promoting DNA damage tolerance (Deans and West 2011, Huang, Liu et al. 2013, Neelsen and Lopes 2015). However, fork reversal could also have pathological consequences and contribute to genome instability. The relevance of fork reversal as a threat to the genome was described relatively recently in the study showing that reversed forks are formed frequently upon the oncogene activation. Oncogene activation induces replication stress through the deregulation of replication origin firing or induction of topological stress created when replication fork collides with transcription complex (Bester, Roniger et al. 2011, Neelsen and Lopes 2015). Fork reversal might also promote genome instability when the four-way junction undergoes unscheduled cleavage by structure-specific endonucleases (Couch, Bansbach et al. 2013, Neelsen, Zanini et al. 2013, Neelsen and Lopes 2015).

Remodelling of replication fork into a reversed fork requires the involvement of multiple factors. Following replication stress, Rad51 recombinase is loaded onto ssDNA regions of uncoupled forks promoting the re-annealing of parental strands and fork reversal (Neelsen and Lopes 2015, Zellweger, Dalcher et al. 2015). It was reported that RAD51 has an essential function at ongoing forks and is strictly required for the reversal of replication fork facing genotoxic stress (Neelsen and Lopes 2015, Zellweger, Dalcher et al. 2015, Berti, Cortez et al. 2020). Chromatin remodelling proteins, such as ZRANB3 and SMARCAL1, are implicated in the reversal of replication fork through their translocase or helicase activity, in PCNA and RPA-dependent manner, respectively (Blastyak, Pinter et al. 2007, Betous, Couch et al. 2013, Neelsen and Lopes 2015). Our studies have shown that fork reversal is also associated with replication fork stalling at R-loops and, in this context, is followed by replication restart (Chappidi, Nascakova et al. 2020). Thus, fork reversal might be a part of the replication restart process at R-loops.

5.2.2 Remodelling of reversed forks to restart DNA replication

In order to restart the DNA synthesis, the reversed fork needs to be resolved, and the structure of a typical replication fork needs to be restored. RECQ1 is a human helicase driving the restart of DNA synthesis. It binds reversed forks and primes their restart by branch migration. A RECQ1-mediated restart of the reversed fork can be inhibited by poly(ADP-ribose) polymerase (PARP1) (Berti, Ray Chaudhuri et al. 2013). As long as DNA lesion or another roadblocks persists, the PARP1 interferes with the replication. It acts as a molecular switch regulating the transition from fork reversal to replication fork restart (Neelsen and Lopes 2015, Zellweger, Dalcher et al. 2015). Another possibility of reversed fork reactivation is mediated by DNA replication ATP-dependent helicase (DNA2) and Werner syndrome ATP-dependent helicase (WRN). The nuclease activity of DNA2 and ATPase activity of WRN promote the regulated restart of the reversed fork by resecting the regressed arms and recruiting the factors promoting restart of DNA synthesis (Berti, Ray Chaudhuri et al. 2013, Neelsen and Lopes 2015, Thangavel, Berti et al. 2015, Zellweger, Dalcher et al. 2015). Once the four-way junction of reversed fork is formed, the activity of multiple proteins is necessary for structure stabilisation and for promoting of the fork restart. One of the essential proteins is ssDNA-binding protein RPA, which protects single-stranded regressed arms of the reversed fork and block their further degradation. Additionally, RPA, coating regressed arms, might be replaced by Rad51, thereby promoting the homology-driven invasion of the re-annealed parent strands, resulting in recombination-mediated fork restart (Neelsen and Lopes 2015, Zellweger, Dalcher et al. 2015). Our studies have shown that the restart of R-loop-stalled forks requires RECQ1 and is stimulated by PARP inhibition or ZRANB3 depletion (Chappidi, Nascakova et al. 2020). These findings suggest RECQ1 promotes the restart of R-loop stalled forks by converting reversed forks back to three-way junction.

5.2.3 Resolution of R-loops-mediated TRC to restart DNA replication and RNA synthesis

Our studies have shown that the restart of DNA synthesis at R-loop-stalled forks requires RECQ5 DNA helicase, MUS81/EME1 endonuclease, RAD52 ssDNA-annealing factor, the DNA ligase IV (LIG4)/XRCC4 complex and the non-catalytic subunit of DNA pol delta (Figure 13) (Chappidi, Nascakova et al. 2020). Our experiments suggested that RECQ5 disrupts RAD51 nucleoprotein filament on the arrested fork to prevent fork reversal and to facilitate fork cleavage by MUS81/EME1. We proposed a model in which fork cleavage by MUS81/EME1 relieves the torsional stress created by converging replisome and RNA polymerase complex. The torsional stress blocks the progression of polymerases and presumably, triggers the formation of R-loops (Garcia-Muse and Aguilera 2016, Hamperl, Bocek et al. 2017, Teloni, Michelena et al. 2019). Then, RNA synthesis could be restored, and the obstacles would be resolved, allowing the restart of replication. We propose that MUS81/EME1-mediated cleavage of stalled forks is followed by religation of fork mediated by LIG4/XRCC4 complex after the parental strands are re-annealed by RAD52. Thus, this fork cleavage-religation cycle would ensures the restart of R-loop-stalled replication forks without disruption of the transcription complex. In support of this model, we found that the restart of R-loop-stalled forks requires active transcription and the presence of the transcription elongation factor ELL (Chappidi, Nascakova et al. 2020). However, it still remains to be determined how R-loop is eliminated to facilitate the putative transcription restart. One of the candidate proteins that could mediate this transaction is the RNA/DNA helicase Senataxin. Of note, the yeast Senataxin homolog Sen1 has be shown to prevent accumulation of R-loops and to promote replication fork progression through highly transcribed genes (Alzu, Bermejo et al. 2012).

5.2.4 Fork reversal and DNA replication restart pathways as a novel target for chemotherapeutic treatments

One of the most frequently used approaches in cancer therapy is killing the cancer cells or arrest their proliferation by targeting DNA replication. Different strategies for replication impairment are usually combined in chemotherapeutic treatments. The candidate replication inhibitors target DNA topoisomerases, the essential enzymes in the resolution of TRCs, with the most famous being camptothecin (CPT), an inhibitor of DNA topoisomerase I (TOP1). CPT is commonly used to treat lung, ovarian and colorectal cancers (Pommier 2013, Zellweger, Dalcher et al. 2015). Our work, as well as others, showed that fork reversal and restart of DNA replication is a global response to different sources of replication stress (Berti, Ray Chaudhuri et al. 2013, Neelsen and Lopes 2015, Zellweger, Dalcher et al. 2015, Di Marco, Hasanova et al. 2017, Chappidi, Nascakova et al. 2020). Thereby, there is a great potential to identify novel targets for cancer chemotherapy based on replication impairment. Interestingly, the cleavage of genomic DNA by the MUS81 endonuclease and PARP-dependent repair pathways were shown to lead to the accumulation of cytosolic dsDNA, which stimulates the STING-dependent production of type I interferons, subsequently leading to the activation of immune response and rejection of the tumour (observed in prostate tumours). MUS81 was characterised as a tumour suppressor, which alerts the immune system and enhances the innate and adaptive anti-cancer immune responses (Ho, Zhang et al. 2016).

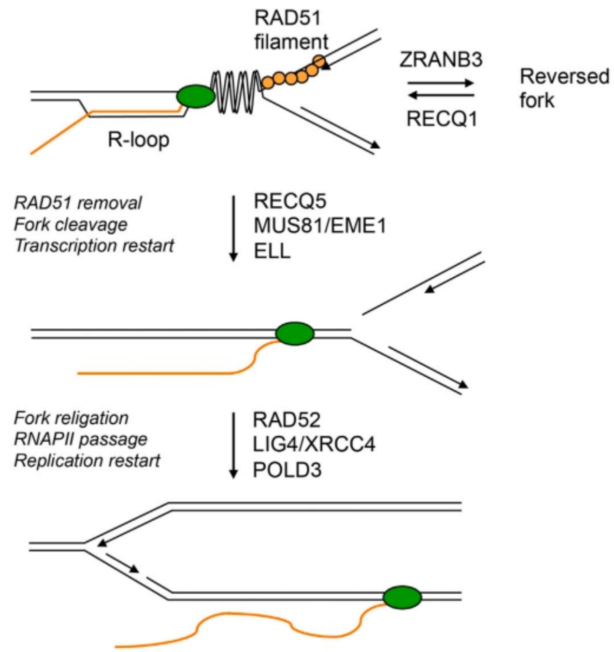

Figure 13: The schematic model of fork cleavage-religation cycle (adapted from (Chappidi, Nascakova et al. 2020)

5.3 Immune escape mechanism of tumour mediated by cGAS-STING pathway

The life of every organism depends on the capability of cells to detect and eliminate any pathogenic entities attacking the organism. In mammals, the anti-microbial and anti-viral defence is carried out by various sensing strategies of the innate immune system. The central strategy involves intracellular signalling receptors, which are able to sense the presence of 'non-self' DNA and initiate signalling cascade, leading to the production of a vast repertoire of immune and inflammatory mediators. Even thought, the canonical pathway of innate immune sensing of 'non-self' DNA has been characterised (Ablasser, Schmid-Burgk et al. 2013, Marcus, Mao et al. 2018, Ablasser and Chen 2019), the question of communication between tumour and non-cancerous cells within tumour microenvironment (TME) has not been answered yet.

5.3.1 Molecular mechanism of the cytoplasmic DNA sensing pathway

The canonical pathway mediating the immune response to 'non-self' DNA, 'self' cytoplasmic DNA or pathogen-derived factors includes DNA-sensing enzyme called cyclic guanosine monophosphate adenosine monophosphate (cyclic GMP-AMP) synthase (cGAS) (Sun, Wu et al. 2013, Wu, Sun et al. 2013, Ablasser and Chen 2019). cGAS is activated upon binding to double-stranded DNA (dsDNA) in a sequence-independent manner. Once the interaction between cGAS and dsDNA has occurred, the cGAS undergoes a structural rearrangement and become active. Activated cGAS converts adenosine 5'-triphosphate (ATP) and guanosine 5'-triphosphate (GTP) into cyclic GMP-AMP (cGAMP) (Gao, Ascano et al. 2013, Ablasser and Chen 2019). Although double-stranded RNA or single-stranded DNA are potential activators of cGAS, neither of the molecules can initiate the structural rearrangement of the catalytic unit essential for cGAS activation, thus are unable to stimulate cGAS-STING sensing pathway. In the next step, cGAMP binds to and activates an endoplasmic reticulum-bound adaptor protein STING (stimulator of interferon genes) (Kato, Omura et al. 2017, Ablasser and Chen 2019). cGAS-STING-mediated response initiates signalling pathway, which stimulates TANK

binding kinase 1 (TBK1, also called NF-κB activating kinase). Subsequently, TBK1 is responsible for regulating innate immunity by a licensing of STING, and recruiting the interferon regulatory factor 3 (IRF3). Another role of TBK1 is to phosphorylate and activate IRF3, which, upon the activation, dimerises and translocates into the nucleus to initiate the transcription of immune responsive genes, including interferons and immunomodulatory genes. The hallmark of a cGAS-STING signalling pathway is the up-regulation of the expression of type I interferons (INF) (Saitoh, Fujita et al. 2009, Liu, Cai et al. 2015, Gonugunta, Sakai et al. 2017, Ablasser and Chen 2019).

5.3.2 The cytoplasmic DNA detecting system: friend or foe?

The cytoplasmic DNA-detecting mechanism is a powerful tool for cells to protect themselves against a massive repertoire of human pathogens. However, the evidence is showing that self-DNA-mediated activation of an immune system possesses a noxious function and is a driving factor of the diverse spectrum of diseases, including Parkinson's disease, myocardial infarction and cancer (King, Aguirre et al. 2017, Cao, Schiattarella et al. 2018, Kerur, Fukuda et al. 2018). The agonist and antagonist of cGAS and STING could become effective therapies for many diseases, including autoimmune and neurodegenerative diseases and cancer (Ablasser and Chen 2019)

5.3.3 cGAS-STING signalling pathway and cancer

Cancer represents an example of pathological disease in which the cGAS-STING-mediated 'self'-DNA sensing plays an essential role in anti-tumour immunity. However, the activation of the 'self'-DNA signalling has a potential to act as an antagonist for anti-tumour immunity, promoting cancer metastasis.

Cancer cells are constantly under stress resulting from harbouring chromosomal abnormalities, genomic DNA damage and hyperproliferation. As a consequence of stress, cancer cells, that harbour genomic DNA damage, often form micronuclei or accumulate fragments of chromatin in the cytoplasm. In addition, the level of dsDNA in cytoplasm further increases upon various DNA-damaging therapies, such as radio- or chemotherapy (Ablasser, Goldeck et al. 2013, Shen, Le Bert et al. 2015, Ablasser and Chen 2019,

Schadt, Sparano et al. 2019). The excessive amount of cytoplasmic DNA is being sensed by cGAS in a cell-autonomous manner. As a result of cGAS activation, stimulation of cGAS-STING signalling pathway promotes the production of inflammatory and immuno-stimulatory molecules, such as cytokines and ligands for natural killer cells, that in return facilitate the elimination of cancerous cells (Fuertes, Kacha et al. 2011, Woo, Fuertes et al. 2014, Ablasser and Chen 2019). There are evidences showing that the cytoplasmic DNA is derived from genomic DNA. Interestingly, the overexpression of RNase H1 decreased the levels of cytosolic DNA and the type I INF production. It suggests that the fraction of cytoplasmic DNA originate from R-loop-mediated genome instability, which is a hallmark of cancer cells (Shen, Le Bert et al. 2015).

Cell-non-autonomous mechanism of cancer cell removal depends on the activity of antigen-presenting cells (APCs), such as macrophages or dendritic cells (Fuertes, Kacha et al. 2011, Woo, Fuertes et al. 2014, Ablasser and Chen 2019). Activated cGAS-STING pathway in APCs, as a response to cancer-cell derived signals, stimulates the production of immune-stimulants, necessary for priming of tumour-specific T cells. Then the primed T cells can be recruited to the tumours and promote their anti-tumour activities (Woo, Fuertes et al. 2014, Corrales, McWhirter et al. 2016).

The immune system plays a critical role in suppressing cancer, especially in the regulation of tumour growth and suppression of cancer spreading. However, the mechanism by which the immune system initiates sensing of tumours and how are immune cells able to differentiate cancer cells from the abundance of normal cells are questions that are poorly understood. Cancer cells have evolved the mechanisms which help them to evade from the immune surveillance system (Binnewies, Roberts et al. 2018, Schadt, Sparano et al. 2019). Various types of cancer cells silence the expression of cGAS and STING, which allows them to escape from the immune control system. Some cancer cells are able to misuse the cGAS-STING pathway for their advantage. For example, brain metastatic cancer cells can activate cGAS-STING signalling in neighbouring astrocytes, stimulate the inflammatory response, which in turn promotes the metastasis of cancer cells (Chen, Boire et al. 2016, Ablasser and Chen 2019). We showed that cancer cell-intrinsic expression of cGAS is a significant determinant of tumour immunogenicity and a potential predictor of cancer prognosis and response to treatments (Schadt, Sparano et al. 2019).

Tumours can be divided into two groups based on the immunogenicity or the level of the immune infiltrates in the TME. The TME could be characterised as a "cold", being T cells-ignorant or a "hot", being T cell-inflamed (Gajewski, Schreiber et al. 2013, Gajewski 2015, Chen and Mellman 2017, Duan, Zhang et al. 2020). The hot tumours usually have a better response to the immunotherapies. Therefore, many studies have focused on converting cold non-inflamed tumours into hot ones to achieve better results (1994, Gajewski 2015, Duan, Zhang et al. 2020). One of the characteristic features of hot tumours is a type I INF signature (Gajewski, Schreiber et al. 2013).

The mechanism of cancer-cell-derived DNA delivery to the APCs, thus promoting the immune response, was poorly understood. The DNA transfer model predicted that the activity of STING, as well as cGAS, are required in APCs. In contrast, our study challenged this model and suggested that it is not a cancer-cell derived DNA, but cancer cell-intrinsic cGAMP, which is delivered into the cytosol of APCs (Schadt, Sparano et al. 2019) to stimulate STING pathway and consequently activate the adaptive immune system (Ablasser, Goldeck et al. 2013, Marcus, Mao et al. 2018, Schadt, Sparano et al. 2019). Our study suggests that the minimal requirements to activate T cells are cGAS expression in cancer cells and STING in hots cells. Additionally, we have shown that cGAMP is transferred via gap junctions from cancer cells to APCs *in vitro* (Schadt, Sparano et al. 2019). The question whether the transfer mechanism is similar in vivo has not been determined yet.

Interestingly, we have also shown that cancer cell-intrinsic production of cGAS has a significant influence on the quality of immune infiltration in human colorectal adenocarcinoma (Schadt, Sparano et al. 2019). The T cell infiltration is often associated with increased survival of cancer patients. We observed that tumour-adjacent non-cancerous tissues express cGAS, whereas the cGAS expression was rare in cancer cells, suggesting that the loss of cGAS expression is an immune escape mechanism promoting the progression of cancer (Schadt, Sparano et al. 2019) as it was detected in case of colorectal cancer (Yang, Huang et al. 2017). Similar results were obtained with patients suffering from *H. pylori*-induced gastric cancer (Song, Peng et al. 2017). The expression of STING protein was strongly decreased in tumour tissues *in vivo*, suggesting that the gastric tumour cells initiated the immune escape mechanism (Song, Peng et al. 2017). It was shown that low STING level correlates with increased tumour size, tumour invasion depth, and reduced

patients' survival. Additionally, STING depletion promoted viability, migration and invasion of gastric cancer cells *in vitro* (Song, Peng et al. 2017). Our study suggested that the cGAS expression by cancer cells might be a robust prognostic biomarker for survival and potential indicator for the response of tumours on therapies (Schadt, Sparano et al. 2019).

6 SUMMARY

1. *Helicobacter pylori* infection is known to induce DNA double-strand breaks that are likely to be a driving source of the transformation of normal gastric cells into pre-cancerous gastric cells. However, the mechanism responsible for DSB accumulation remained to be elucidated. We have shown that:

   - *H. pylori*-induced DSBs depend on the intact ALPK1/TIFA/NF-! B pathway and transcription of NF-! B target genes
   - *H. pylori*-induced DSBs result from replication stress caused by transcription-replication conflicts associated with the formation of R-loops
   - R-loop-induced replication stress in *H. pylori*-infected cells depends upon the ADP-heptose/ALPK1/TIFA/NF-! B signalling axis

2. Unresolved collisions between replication and transcription are linked to replication stress and genomic instability. How R-loop-stalled replication fork is restarted and what is the fate of the transcription complex is unknown. We have shown that the restart of R-loop-stalled forks:

   - is triggered by RECQ5-mediated suppression of fork reversal
   - is mediated by cleavage of the stalled fork with MUS81/EME1 endonuclease and subsequent fork religation by LIG4/XRCC4
   - is dependent upon reactivation of transcription

3. The stimulation of immune system to fight against the tumour is triggered by cGAS/STING signalling axis. What is a signal for immune cells to recognise and infiltrate the tumour and how is the tumour-derived signal delivered to the immune system is not clear. We have shown that:

   - cancer cell-derived cGAS and host cell-derived STING are required for immune response to the tumour
   - cancer cell-intrinsic cGAMP is transferred into the host cells
   - cGAMP is transferred *via* tight junctions into the host cell in TME

- cGAS expression by cancer cells improves the tumour immunogenicity and response to therapies.

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8 PUBLICATIONS