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Bacteria and Cancer

From toxin delivery to carcinogenesis

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BACTERIA AND CANCER: FROM TOXIN DELIVERY TO CARCINOGENESIS

Riccardo Guidi

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Abstract

Epidemiological evidence link certain chronic bacterial infections to a higher risk of cancer development. Induction of an inflammatory circuit and the accumulation of genomic instability are considered mechanisms by which bacteria contribute to malignant transformation. Whether production of toxins, that directly induce DNA damage, enhances the tumor promoting effects of chronic inflammation is still unknown. This thesis investigates the role of the cytolethal distending toxin (CDT), the first bacterial genotoxin identified, in carcinogenesis. We have studied the cellular responses to acute and chronic CDT intoxication, as well as the toxin production and secretion during bacterial infection.

Acute CDT intoxication triggers the activation of the DNA damage response and induction of survival signals in the target cells, which may favor cancer growth. Through a screening of a *Saccharomyces cerevisiae* library, we identified 78 genes whose deletion confers hypersensitivity to CDT exposure (paper I). Bioinformatics analysis revealed that DNA repair and endocytosis were the two most represented signaling pathways among the genes identified in the screening. We further demonstrated that in response to DNA damage, the flap-endonuclease 1 (FEN1) regulated the RHOA-dependent activation of the actin cytoskeleton and cell survival via the ROCK and MAPK p38 kinases, respectively, revealing a complex and previously unrecognized crosstalk between DNA damage, cell survival and cytoskeleton dynamics.

As chronic exposure to DNA damaging agents is a well-characterized risk for cancer development, we assessed the effects of chronic CDT exposure *in vitro* (paper II). Cells grown for more than six months in the presence of sub-lethal toxin doses showed an altered DNA damage response, genomic instability, and acquisition of several hallmarks of tumor progression, such as enhanced oxidative stress and capacity of anchorage independent growth. Cell survival of the chronically intoxicated cells was dependent on sustained activation of the MAPK p38 pathway. To dissect the role of CDT in tumor development *in vivo*, we produced a *Salmonella typhimurium* strain that encode for the *Salmonella typhi* CDT-like toxin, known as typhoid toxin (TT). As control, we used an isogenic strain carrying an inactive toxin. Both strains successfully infected the immunocompetent sv129 mice for more than 2 months, however only the bacteria expressing the active genotoxin caused an enhanced inflammation in liver and spleen.

To understand how this potential bacterial carcinogen is delivered to the target cells, we studied the secretion of the *Salmonella* TT (paper III). We demonstrated that TT is secreted from the bacterium via outer membrane vesicles (OMVs). These vesicles are further released into the extracellular environment via an exocytosis-like process. The paracrine internalization of TT-loaded OMVs by bystander cells was dependent on dynamin-1-mediated endocytosis.

Taken together, our studies contribute to elucidate the survival strategy of cancer cells in response to CDT, its role in cancer progression and its secreting mechanisms.

PAPER I

J. Cell Sci. 2011 Aug; 124:2735-42

Bacterial genotoxin triggers FEN1-dependent RhoA activation, cytoskeleton remodeling and cell survival

Guerra L[1]*, Guidi R[1]*, Slot I[1], Callegari S[1], Sompallae R[1], Pickett CL[2], Åström S[3], Eisele F[4], Wolf D[4], Sjögren C[1], Masucci MG[1], Frisan T[1]

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*shared authorship

PAPER II

Cell. Microbiol. 2013 Jan; 15: 98-113

Chronic exposure to the cytolethal distending toxins of Gram-negative bacteria promotes genomic instability and altered DNA damage response

Guidi R[1]*, Guerra L[1]*, Levi L[1], Stenerlöw B[2], Fox JG[3], Josenhans C[4], Masucci MG[1], Frisan T[1].

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PAPER III

Cell. Microbiol. 2013 Jul; 15: 2034-2050

***Salmonella enterica* delivers its genotoxin through outer membrane vesicles secreted from infected cells**

Guidi R[1], Levi L[1], Rouf SF[2], Puiac S[2], Rhen M[2], Frisan T[1].

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2- Departments of Microbiology, Tumor and Cell Biology, Karolinska Institutet, Stockholm, Sweden

Other publications

PLoS One 2010 Jan; 5:e8924

Myc is required for activation of the ATM-dependent checkpoints in response to DNA damage

Guerra L[1], Albihn A[2], Tronnersjö S[2], Yan Q[2], Guidi R[1], Stenerlöw B[3], Sterzenbach T[4], Josenhans C[4], Fox JG[5], Schauer DB[5], Thelestam M[1], Larsson LG[2], Henriksson M[2], Frisan T[1]

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Toxins 2011 Mar; 3:172-90

The biology of the cytolethal distending toxins

Guerra L, Cortes-Bratti X, Guidi R, Frisan T

Departments of Cell and Molecular Biology, Karolinska Institutet, Stockholm, Sweden

FEBS J. 2011 Dec; 278:4577-88

Do bacterial genotoxins contribute to chronic inflammation, genomic instability and tumor progression?

Guerra L, Guidi R, Frisan T

Departments of Cell and Molecular Biology, Karolinska Institutet, Stockholm, Sweden

Bacterial Pathogenesis
(Edited by Locht C and Simonet M, Caister Academic Press 2012)

Toxins acting on intracellular targets: only foes or also friends? (chapter)

Frisan T, Guidi R, Guerra L

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Objectives

Numerous evidence link chronic bacterial infections to cancer. The human bacterium *H. pylori* was found to cause peptic ulcers and to predispose individuals to gastric carcinomas, while chronic carriers of *Salmonella enterica* serovar Typhi are at higher risk of liver cancer. More recently, certain commensal bacteria of the intestine were shown to contribute to inflammatory bowel disease (IBD), a condition that predispose to colorectal adenocarcinomas.

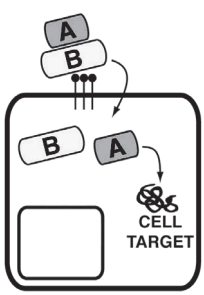
This work aims to dissect the molecular mechanisms underling bacterial-induced carcinogenesis. Particularly, we presents studies on a bacterial toxin secreted by several Gram-negative pathogens, which presents potential carcinogenic characteristic.

1 A BACTERIAL GENOTOXIN

The *repertoire* of bacterial toxins comprises more then 300 members, including natural variants, isoforms and seriotypes. Toxins exert a myriad of functions, hijacking the cellular machinery of the host to help the pathogen to establish a successful infection.

Almost all bacterial toxins target the plasma membrane or cytosolic molecules, with the exception of two: Colibactin and Cytolethal Distending Toxins (CDTs). These toxins enter the nucleus of target cells to cause DNA damage, and due to their characteristics, Colibactin and CDTs are the only two "bacterial genotoxins" described[1].

This thesis presents three studies on the biology of CDTs. We characterized the cellular response that follows acute CDT intoxication (paper I), CDTs carcinogenic properties *in vitro* and *in vivo* (paper II and preliminary data) and CDT expression and secretion from the cancer-associated pathogen *Salmonella enterica* (paper III).



BOX.1: Bacterial toxins have A- and B-domains that exert different functions: A-domais target a component of the cell, while B-domains mediate receptor binding and internalization.

Structure of Cytolethal Distending Toxins

Bacterial toxins are secreted from the bacterium in the extracellular environment, from where they bind to the membrane of target cells and triggers their active endocytosis. To exert this function, toxins present a conserved organization: the A and B domains (Box.1). A-domains are the catalytic subunits of toxins, while B-domains are "assistant" that mediate the toxin binding and internalization. Bacterial toxins can be formed by different stoichiometry of A and B domains. Diphtheria Toxin has one catalytic and one binding domain (A-B)[2], Cholera Toxin has five identical B domains (A-B5)[3,4], and Anthrax Toxin has two distinct catalytic domains (A2-B)[4].

The Cytolethal Dystending Toxins (CDTs) is a family of toxins produced by Gram-negative bacteria, notably *Escherichia coli*[5,6], *Aggregatibacter actinomycetemcomitans*[7], *Haemophilus*

Bacteria	Designation	Reference
Escherichia coli	EcCDT-I	Scott and Kaper, 1994
	EcCDT-II	Pickett et al., 1994
	EcCDT-III	Peres et al.,
	EcCDT-III-9063/02	Bielaszewska et al.,2004
	EcCDT-IV	Toth et al., 2003
	EcCDT-V	Janka et al., 2003
	EcCDT-V-9282/01	Bielaszewska et al.,2004
	EcCDT-V-5249/01	Bielaszewska et al.,2004
	EcCDT-V-2996/96	Bielaszewska et al.,2004
Escherichia albertii	EaCDT	Hyma et al., 2005
Shigella dysenteriae	SdCDT	Okuda et al., 1995
S. boydii	SbCDT	Hyma et al., 2005
S. sonnei	SsCDT	Okuda et al., 1995
Salmonella enterica Typhi	STCDT	Haghjoo and Galan, 2004
Campilobacter jejuni	CjCDT	Pyckett et al., 1996
C. coli	CcCDT	Asakura and Yamasaki, unpubl.
C. fetus	CfCDT	Asakura and Yamasaki, unpubl.
C.hyointestinaris	ChCDT	Samosornsuk et al., unpubl.
C. lari	ClCDT	Pickett, 1996
C. upsaliensis	CuCDT	Pickett, 1996
Haemophilus ducreyi	HdCDT	Cope et al., 1997
Actinobacillus actinomycetem.	AaCDT	Sugai et at., 1998
Helicabacter pullorum	HpCDT	Young et al., 2000
H. flexispira	HfCDT	Kostia et al.,2003
H. cinaedi	HcCDT	Taylor et al., 2003
H. hepaticus	HhCDT	Young et al., 2000
H. bilis	HbCDT	Chien et al., 2000
H. canis	HcCDT	Chien et al., 2000
Helicobacter spp.	HspCDT	Chien et al., 2000

Table.1: List of Gram-negative bacteria found to encode for CDT genes. In red: human pathogens.

ducreyi[8], *Shigella* spp.[9], *Campylobacter* spp.[10], *Helicobacter* spp.[11], and *Salmonella enterica* serovar Typhi[12] (Table.1). The presence of CDT in pathogens with very different clinical outcomes (from gingivitis to typhoid fever) underlines its biological relevance, though limited evidence support its role as virulence factor during bacterial infection. CDTs are A-B2 toxins composed of three subunits: CdtA, CdtB and CdtC (Fig.1a). CdtB is the A-domain, while CdtA and CdtC are B-domains. As all three subunits are essential to form a fully active toxin, CDTs genes are often found in a single, horizontally acquired operon[13].

CdtA and CdtC are structurally similar to each other and to the ricin toxin B-domain. Ricin is a potent ribosome-inhibitor plant toxin, and its B-domain binds to sugar moieties present on the surface of cells[14]. Similar to ricin B-domain, CdtA and CdtC binds to polysaccharides on the cellular surface, though with different strength and specificity[15]. Moreover, CdtA and CdtC seem to have distinct functions. CdtA presents a patch of aromatic amino-acids that tightly binds to glycoproteins and glyocosphingolipids[15], and when mutated, it abolish CDT toxicity

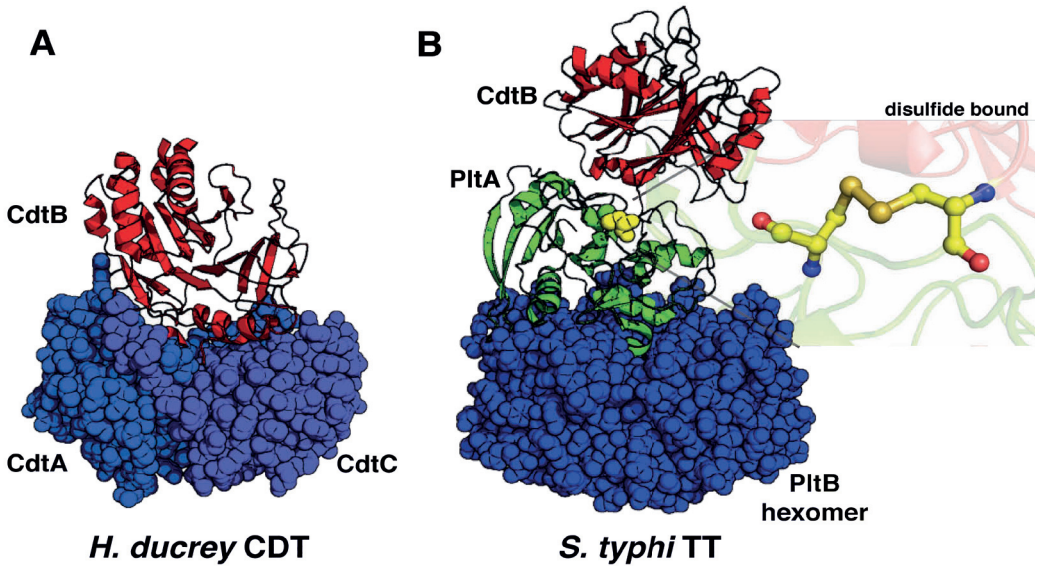
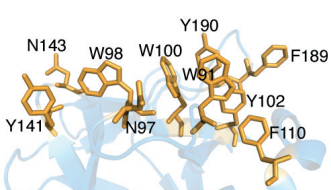


Fig.1: Crystal structures of canonical CDT (A) and *S. typhi* Typhoid Toxin (B). Inset: the unique disulfide bound of typhoid toxin.

in vitro[16] (Box.2). On the other hand, CdtC seems to assist the active subunit to correctly internalize in cells and to reach the nucleus[17]. CdtB is structural homologue to mammalian DNase I (Fig.2). This class of enzymes breaks the phosphodiester bounds of the DNA backbone, triggering a DNA damage response[18,19]. Mutations of the CdtB catalytic domain and the Mg^{2+} binding domain abolish CDTs intoxication[19]. In absence of CdtA and CdtC, ectopic expression of CdtB in yeast, or microinjection in HeLa cells, causes DNA damage, arrest and eventually apoptosis[18,20,21].



BOX.2: The CdtA domain presents ten aromatic aminoacids that mediate binding to cellular receptor(s)

Cellular internalization and effects

Upon binding to a yet unknown cellular receptor, CDTs are internalized by endocytosis[22] and enters the early endosome, from where cargoes can follow different pathways: i) be send back at plasma membrane, ii) be degraded into lysosomes, and iii) access the Golgi and the endoplasmic reticulum (ER). *Hemophilus ducrey* CDT (HdCDT) is internalized in a dynamin-dependent manner, and enters the retrograde pathway from the Golgi to the ER compartment[23,24]. The mechanisms by which CDTs are then transported into the nucleus remains to be characterized[25]. To date, most studies

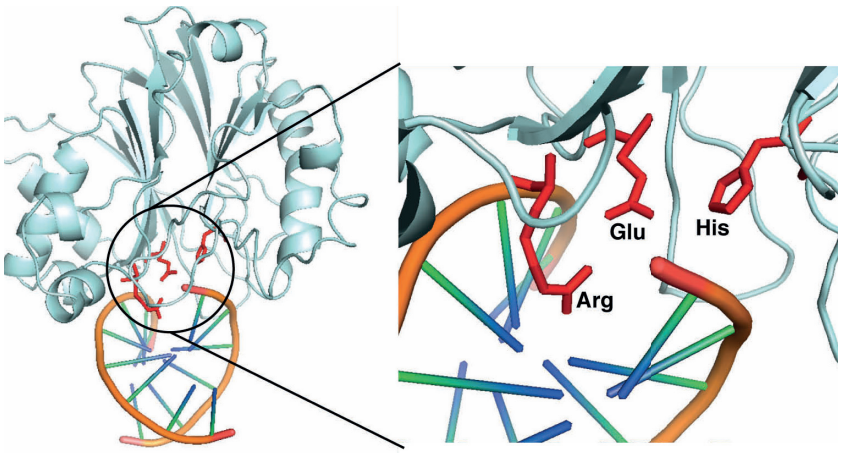
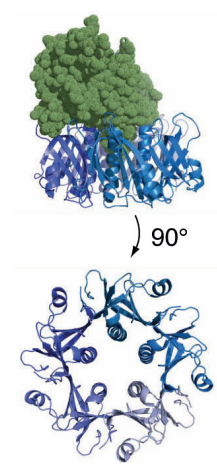


Fig.2: The binding of mammal DNAase I to the DNA. Inset: the catalytic pocket.

have looked at the mechanisms of CDTs internalization with recombinant purified toxins. It is worth notice that CDTs from *E. coli* and *C. jejuni* were found secreted within vesicles released from the bacterial outer membrane, the outer membrane vesicles (OMVs)[26,27].

Typhoid Toxin is a particular type of CDT

Salmonella typhi encodes for a different version of CDTs, named Typhoid Toxin (TT) (Fig.1b). The TT operon is in a region of *Salmonella* chromosome distinct from the pathogenicity islands SPI-1 and SPI-2, probably also horizontally acquired. Whole genome analysis of *Salmonella* showed that the TT operon was absent in *S. bongori* and *S. enterica* serovar Typhimurium[12], thus making this toxin unique for the human-restricted pathogen *S. typhi*. TT is composed of three subunits: *S. typhi* CdtB (StCdtB), pertussis-like toxin A and pertussis-like toxin B (PltA and PltB). PltB functions as B domain, and is assembled in a ring-like hexomer that interacts with cellular receptors (Box.3). PltA is a functional ADP-ribosylase with high homology to the pertussis toxin S1 domain, but its cellular target is unknown[12]. Lastly, StCdtB is a typical cytolethal distending toxin A domain, with similar catalytic pockets and mode of action[12]. TT is the first A2B5 toxin described with two distinct A-domains linked together via a disulfide bound[28](Fig.1b, detail). Mutation in any of the two cysteine residues that mediate the bound prevents intoxication of cells infected with *S. typhi*[28] but it is dispensable for the assembly of the toxin *in vitro*[29].



BOX.3: PltB forms an hexomer platform that interacts with PltA.

In spite the presence of two A-domains, TT seems to rely primarily on its DNase activity to exert toxicity. Mice injected with the wild-type or the PltA-inactive TT, experience comparable loss of weight and death within a week, while StCdtB-inactive TT causes no symptoms. Though i.v. injection of TT in C57BL/6 mice does not cause fever, the toxin alone is able to reduce peripheral blood leukocytes, particularly neutrophils, a characteristic of *S. typhi* infections in humans[28].

Since the primary effect of CDT in the target cells is induction of DNA damage, I will discuss the DNA damage response, before we consider the cellular effects of intoxication and whether CDT may contribute to the tumorigenic process.

2 THE DNA DAMAGE RESPONSE

As insults like oxidative stress, irradiation and other genotoxin agents can threaten genome integrity, multicellular organisms has evolved a complex molecular machinery that blocks cellular proliferation while try to fix the damage. This complex network is called the DNA damage response (DDR).

CDTs cause DNA damage in both yeast and human cell lines[21,30] and trigger a DDR similar to that evoked by ionizing irradiation (IR), a well-known carcinogen[20,31]. IR is energy transmitted via x- and γ- rays or α- and β-particles, and similar to CDTs, causes DNA double-stranded brakes (DSBs)[30].DSBs can be repaired following three distinct pathways: homologous recombination (HR), non-homologous end-joining (NHEJ), and alternative NHEJ (alt-NHEJ)(Fig.3).

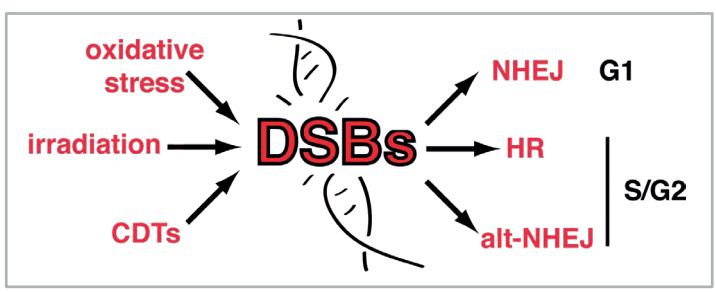


Fig.3: Different genotoxin agents can cause double strand breaks (DSBs) to the DNA, and the cell can repair the damage following alternative pathways.

These three repair mechanisms involve different proteins and ensure different degrees of fidelity after the damage is repaired. In general, the choice upon which DDR to execute depends on the phase of the cell cycle. HR is favored in S/G2 phase, as this pathway requires an homologous template from which to copy an entirely new DNA fragment[32]. On the other hand, NHEJ and alt-NHEJ do not require DNA templates, and are prevalent in G1[33].

Regardless the pathway, DDR follows three steps: i) sensing the presence of damaged DNA, ii) propagate the alarm signals and iii) repair the harm. Proteins classified as sensors, mediators and effectors accomplish each one of these steps. Importantly, mediators also activate different cyclin inhibitors, which block the

cell cycle progression.

Sensing and repairing the damage

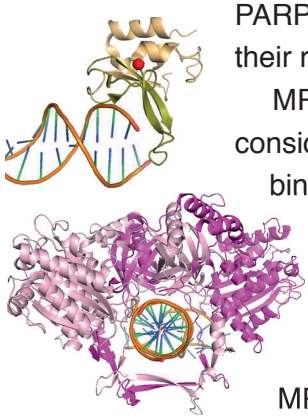
SENSORS
feel the damage

MEDIATORS
propagate the signal

EFFECTORS
execute the repair

PARP1/2 and Ku70/Ku80 are important sensors of DNA damage (Box.4). PARP1 and PARP2 belong to the poly(ADP-ribose) polymerase (PARP) family, and initiate the homologous recombination repair[34]. Recruitment of PARP1/2 at the site of DSBs causes a conformational change that activates its enzymatic pocket and attach polyADP-ribose chains (pADPr) on target proteins[35]. The pADPr chains function as a scaffold for other DDR proteins. These include: the sensor/mediator MRN complex (see below), the cell-cycle regulator TP53 and the *ataxia telangiectasia* mutated protein ATM[36].

The Ku70/Ku80 is a protein dimer with a toroid shape that fits into DSBs like a ring around its finger to stabilize its loosing ends[37]. Ku70/Ku80 recruits the DNA-PK catalytic subunit (DNA-PKcs), a mediator that orchestrates NHEJ[38]. In summary, PARP1/2 and Ku70/Ku80 compete for binding the break, and their regulation can influence the choice of the repair[39,40].



BOX.4: Crystal structures of PARP1 (top) and the Ku/70/Ku80 complex (bottom) bound to DNA

MRN and the replication protein A (RPA) complexes are also considered DNA damage sensors, since they are able to directly bind damaged DNA (MRN) or free single strand DNA (ssDNA) during replication fork stalk (RPA)[41,42]. The MRN is a heterocomplex composed by the ATPase RAD50, the exo-endonucleases MRE11 and NBS1. RAD50 binds to DNA ends in its active form (ATP-bound), aggregating MRE11 and NBS1 at the site of the damage[43]. NBS1 has the essential task to recruit the mediator ATM, which propagates the DDR signal and promotes repair[42]. The RPA complex is composed by RPA1/2/3. RPA binds onto exposed ssDNA and signals the presence of a stalked replication fork, leading to the recruitment of the mediator ATR[41]. A schematic representation of the DDR that follows DSBs is depicted in Figure 4.

Following DSBs, ATM phosphorylates the histone 2AX (γ-H2AX) which is important in the propagation of the DDR signal, and protein necessary for repairing the damage (effector proteins, such as CtIP) and inducing cell-cycle arrest (via checkpoint kinase 1 and 2 (CHK1 and CHK2)).

Upon ATM-mediated phosphorylation, CtIP recruits BRCA1[44]

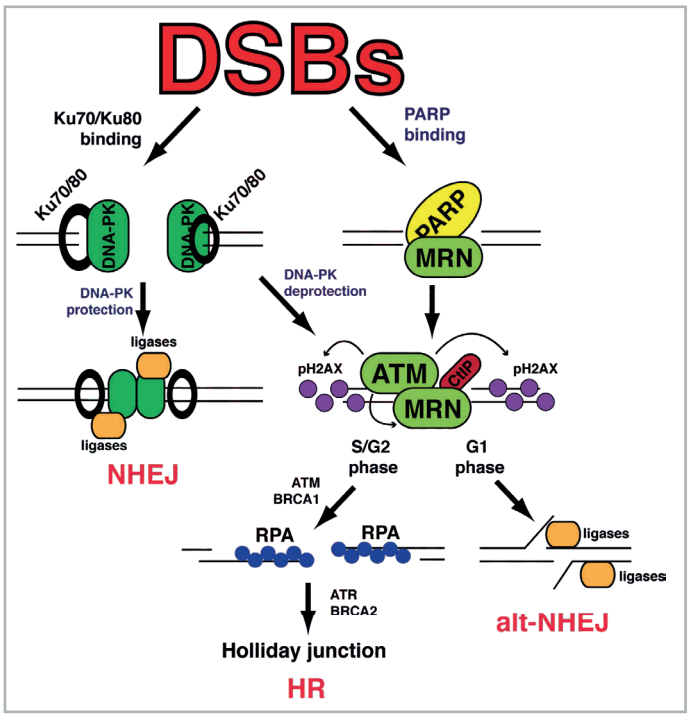


Fig.4: Schematic representation of DNA damage response that follows DNA DSBs.

and leads to an extensive resection of the DNA. RPA1 can now feel the ssDNA and execute the Holliday junction together with BRCA2[36]. In G1 phase, where sister chromatin is missing, CtIP carries on a limited resection (independently from BRCA1) and repairs the damage via the alt-NHEJ pathway[45].

Additionally to repair, ATM is required to activate CHK2, which blocks the cell-cycle progression via the inhibitory phosphorylation of CDC25. In G2/M, CDC25 alone can mediate arrest, but for a proficient G1/S checkpoint, the activity of TP53 is also required. TP53 mediates the transcription of the cycling inhibitor p21, which is essential for a prolonged G1 arrest[46](Fig.5).

CDC25 is a group of phosphatases required for cyclin-dependent kinases (CDKs) activity.

DDR response in CDT intoxicated cells

Cells intoxicated with CDTs shows a similar DNA damage response to that evoked by ionizing radiations, a well know carcinogen[22], with ATM phosphorylation and the activation of the classical DNA damage and checkpoint responses[1]. The DDR signal is further transduced to the cytoplasm via the export of the guanine exchange factor NET1 from the nucleus[30,47], leading to the activation of the small GTPase RHOA[30,47,48]. From here, the NET1-RHOA signal cascade diverges into two different

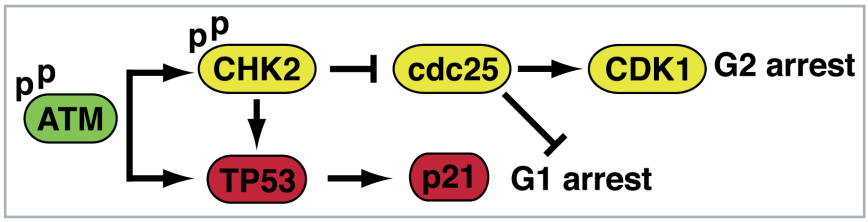


Fig.5: ATM phosphorylates CHK2, leading to cell cycle arrest in G1/S and G2/M (via the CHK2-dependent phosphorylation and inhibition of CDC25) and sustained G1 arrest (via the TP53-dependent transcription of the CDK inhibitor p21). In cell lines carrying an inactive TP53, CHK2 mediates preferentially a G2 arrest.

pathways. On one side, the kinases ROCK1 and ROCK2 induce the formation of actin stress fibers; on the other side, the phosphorylation of the mitogen activated protein kinases p38 (MAPKs p38) promote cell survival[47]. A schematic representation of this pathway is depicted in Figure 6.

In cells of epithelial and mesenchymal origin, acute DNA damage leads to senescence[49], while cells of lymphoid origin die of apoptosis[31]. Interestingly, this cell dependent behavior correlates with the activation of the NET1-RHOA pathway. Understanding the molecular mechanisms by which cells escape CDT-dependent apoptosis might shade light on the possible role of the toxin as carcinogen. Additionally, as chemotherapeutic drugs and irradiation are standard procedures in cancer treatment, these studies might help to refine medical interventions.

PAPER I

To further characterize the survival signals triggered by the NET1-RHOA axis, in paper I we screened for genes whose deletion causes enhanced apoptosis after acute intoxication. To this end, we transfected a *Saccharomyces cerevisiae* mutant library that carry single deletions in nonessential genes with an expression vector encoding for the CdtB gene under an inducible promoter. With this tool, we identified 78 genes whose deletion confers hypersensitivity to the toxin. Gene ontology analysis identified DDR, and more surprisingly endocytosis, as the two most represented pathways among the gene identified in the screening. A similar pattern was previously observed in yeast screening assessing the hypersensitivity to agents that cause DNA DSBs[50,51]. As our primary interest was the RHOA-mediated survival, we used bioinformatics to create a network of all known RHOA primary and secondary interacting partners, and interrogated this database for the presence of gene products found in our analysis. The genes encoding for FEN1 and TSG101

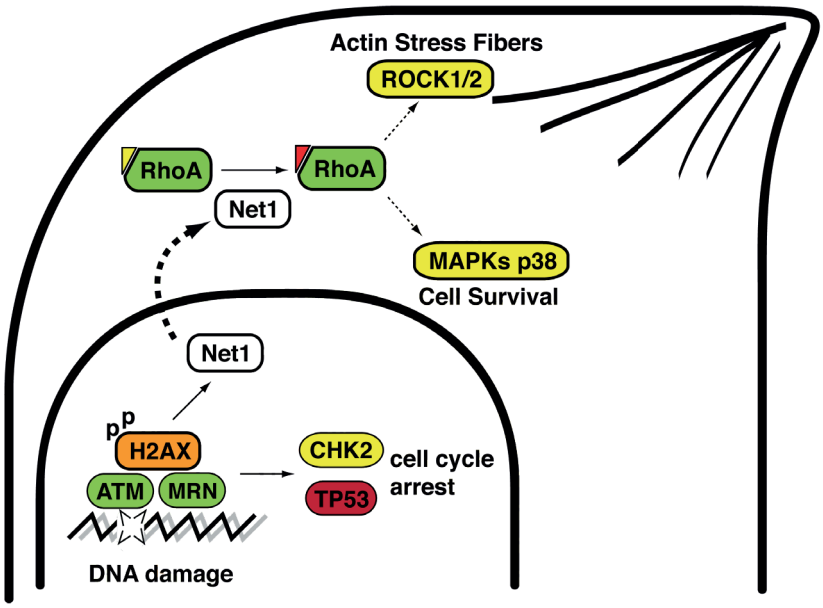


Fig.6: The NET1-RHOA dependent cell survival pathway that is activated in response to acute CDT intoxication.

regulates DDR and endocytosis, respectively, and were further validated in mammalian cells. We demonstrated that FEN1, but not TSG101, regulated RHOA activation, actin stress fibers, MAPK p38 phosphorylation and cell survival in response to DNA damage.

The flap-endonucleases 1 (FEN1) is a multifunctional enzyme with important roles for the normal DNA metabolism[52-55]. FEN1 resolves Okazaki fragments during DNA replication and it is required for functional base-excision repair (BER)[56]. This protein is subjected to different post-translational modification events, including phosphorylation by CDK1 and CDK2[57], which increases during S-phase and enhances FEN1 activity, leading to S to G2 transition. Mutations of one of FEN1 phosphorylation sites (S187) prolong the S-phase in the normal course of the cell cycle[58]. Since FEN1 plays a role in HR[59], its expression is further up-regulated following DNA DSBs in yeast [54].

Our work shows for the first time a role of FEN1 in the activation of the RHOA survival pathway that follows DNA DSBs. In line with our findings, MEFs with a partially inactive FEN1 are also more susceptible to apoptosis following UV irradiation compare to wild-type cells[60].

How can FEN1 control RHOA activity? FEN1 was found to interact with the RHOA-regulating protein ARHGDIA in a yeast two-hybrid screening[65]. The nature of this interaction and its functionality has not been explored yet in mammal cells. As ARHGDIA maintains RHOA in an inactive state[66], it is feasible that the increased transcription of FEN1 following DSBs might be necessary to sequester ARHGDIA. Interestingly, other DDR proteins were found to exert pro-survival function via their interaction with cytoplasmic partners. The DDR sensor DNA-PK can localize in lipid raft microdomains of the plasma membrane[61], where it phosphorylates AKT[62], a pro-survival factor for cancer cells[63]. Similarly, the Ku70/Ku80 protects cells from apoptosis suppressing BAX translocation in the mitochondria[64]. To date there are no report on the presence of FEN1 in the cellular cytoplasm, however, a new truncated FEN1 isoform (generated by alternative translation) was recently found in mitochondria[67].

Ultimately, FEN1 could be important for the activation and translocation of NET1, the nucleotide exchange factor that activates RHOA after CDT intoxication, or for the activation of other components of HR that mediate RHOA activation.

So far I described the importance of survival signals that play a key role in tumorigensis, but alone are not sufficient for cancer initiation/progression. Cancer cells not only have to avoid DNA-damage dependent cell death, but will have to sustain their independent proliferation, become immortal, promote angiogenesis, reprogram their metabolisms and metastasize[68]. The magnitude of genetic rearrangement necessary to accomplish these tasks is achieved via acquisition of genomic instability.

3 GENOMIC INSTABILITY AND CANCER

Genomic instability (GI) is a status in which cells experience genetic rearrangements, which comprises generation of new point mutations, small deletions/insertions of DNA sequences and gross chromosomal translocations[69]. Epidemiological and experimental evidence indicate that GI is a driving force of cancer[70].

How does GI occur?

GI occurs when a chronic, sub-lethal dose of DNA breaks exceed the capacity of the DNA damage response (DDR) to properly repair the damage. The source of DNA damage can be endogenous like a sub-optimal DDR, oncogene activation or oxidative stress; or exogenous, like chemicals, irradiation, or bacterial toxins.

A. Sub-optimal DNA damage response

In hereditary cancer, the origin of GI is attributed to mutations in "caretakers genes", responsible for the correct DNA repair pathway and the mitotic checkpoint[46,71]. For example, germ-line mutations in the DNA mismatched repair protein MSH2[72], or DNA base excision repair gene *MYH*[73] are associated with a higher risk of colon adenomas in humans. Mutations in other DNA repair genes, like breast cancer susceptibility 1 and 2 (*BRCA1/2*), *RAD50*, Nijmegen breakage syndrome 1 (*NBS1*), bloom syndrome helicase (*BML*), Fanconi anaemia genes, and nucleotide-excision repair genes can all predispose to various diseases and tumors, notably leukemia, lymphomas, breast, ovarian and skin cancers[74-77]As these genes encode for proteins with not-redundant roles, their inactivation irreversibly compromises both the repair of DNA and the activation of the checkpoint response.

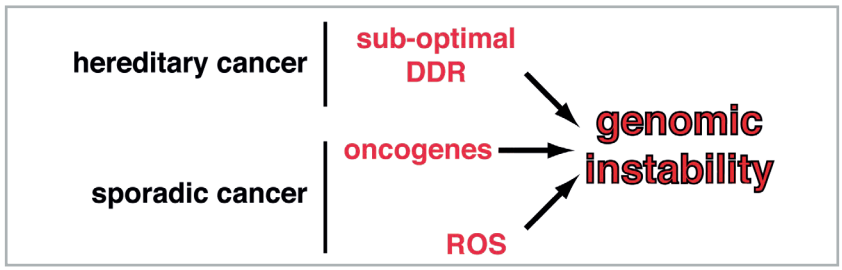


Fig.7: Different mechanisms lead to genomic instability in hereditary and sporadic cancers

B. Oncogene activation

Inactivation of caretakers genes do not seem the main mechanism for acquisition of GI in sporadic cancer[70], suggesting the existence of alternative events, such as oncogene-induced replicative stress[78].

Gross chromosomal abnormalities are seen in murine fibroblasts NIH 3T3 transfected with *HRAS*[79]. The same is true for the immortal rat fibroblasts Rat1A over-expressing *c-MYC*, which present aneuploidy and have dicentric chromosomes[80,81]. Further more, a brief over-expression of *c-MYC* can increase the level of reactive oxygen species and mitigate the TP53 activity in primary human fibroblasts[82], which can become aneuploid[80].

C. Reactive oxygen species (ROS)

Another source of GI might be the exposure of reactive oxygen species or reactive nitrogen intermediates (ROS and RNI) beyond their physiological levels[83]. Oxidative DNA damage is detected by the presence of 8-Oxo-2'-deoxyguanosine (8-oxodG), an oxidated product of the base guanosine. This oxidation further leads to mismatch 8-oxodG with adenine, resulting in a G->T or A->C substitutions[84]. Due to its genotoxic activity, ROS might facilitate cellular transformation. Mice embryo fibroblasts (MEFs) briefly treated with exogenous ROS have enhanced transformation capacity *in vitro* and *in vivo*[85], and genetic alterations that enhance ROS production promote malignant transformation in other cell models[86-88].

Signs of ROS-damage are also seen in inflammatory diseases that precede cancer. Livers of chronic hepatitis patients present elevated levels of 8-oxodG compared to matching controls[89] and transgenic mice with chronic hepatitis progressively accumulate 8-oxodG in their hepatocytes[90]. A persistent oxidative stress may be responsible for the genomic instability seen in primary

bone marrow cell cultures[91], and high level of mitochondrial-produced ROS triggers GI in lymphomas cell lines[92]. Particularly in lymphomas, GI was associated with an increased number of drug-resistant cells[92].

Other examples come from oncogenic viruses. The chromosomal rearrangements observed in primary B-cells infected with the transforming Epstein-Barr Virus (EBV)[93] may be ROS-dependent, as the use of scavengers in EBV-transformed lymphocytes reduces DNA damage[94] and restores telomers function[271].

As I will describe in Chapter 4, also chronic bacterial infections promote ROS production by immune cells at the site of the infection, as means of anti-bactericidal activity.

GI can activate oncogenes

The random DNA mutations caused by GI can hit oncogenes that control cellular proliferation. *RAS*, *CTNNB1* (catenin β1), and *c-MYC* are frequently mutated in human tumors, and their aberrant activation has been proven to be a driving force for tumorigenesis.

Somatic activation of *KRAS* promotes lung cancer in transgenic mice[95], and when genetically activated in the intestine, β-catenin promotes spontaneous polyposis and adenomas[96]. Over-expression of *c-MYC* in the hematopoietic lineage predispose mice to highly penetrant lymphomas and leukemias[97], and *c-MYC* inactivation causes tumor remission[98]. Oncogenes can also work together to facilitate tumorigenesis. Co-expression of *RAS* and *c-MYC* can more easily transform mouse NIH 3T3 fibroblasts *in vitro* than when used individually[272], and the same oncogene combination greatly enhances tumor susceptibility in mice[99]. Ultimately, the success of oncogene-inhibitory drugs that target BCR-ABL, c-KIT and EGFR underlines the key role of oncogenes in cancer development and their therapeutic potential.

Human cells were proven more resistant than mice or chicken to transformation by expression of single or multiple oncogenes, arguing against the role of sole oncogenes in the development of human cancers[273]. GI must therefore subvert also additional mechanisms that block tumor development.

GI can suppress the tumorigenic barrier

The presence of moles on our skin indicates that sporadic proliferation of cells can occur rather frequently in humans[100]. As these melanocytes will rarely become a skin cancer, mammals must have evolved mechanisms that efficiently counteract oncogene-triggered tumors. When human primary fibroblasts are infected with oncoviruses, a rapid cellular proliferation is shortly followed by permanent cell-cycle arrest, called senescence[101], while introduction of *c-MYC* in immortal rat fibroblasts causes massive apoptosis[102]. The activation of these two responses is defined as tumorigenic barriers, a defense mechanisms against de-regulated proliferation. Activation of the tumorigenic barrier reduces cellular transformation *in vitro* and prevents pre-carcinogenic lesions to evolve into fully established cancers *in vivo*[103,104].

Senescence is an irreversible cell-cycle arrest, characterized by cellular distention and activation of markers like β-galactosidase both *in vitro* and *in vivo*[105]. Apoptosis is a programmed cell death that, at least in response to stress signals caused by hyper-proliferation, is characterized by cytochrome-c release and caspases activation[106].

In the contest of cancer development, the tumorigenic barrier can be activated by oncogene-induced replicative stress[78,107]. Replicative stress is caused by the over-expression of oncogenes like *c-MYC* and *HRAS* that cause the simultaneous firing of the same DNA origin of replication, causing an increase of fork stalling[108,109]. The consequent activation of DDR triggers a signal cascade that leads to a TP53-mediated activation of the tumorigenic barrier[110]. Signs of oncogene-induced DDR and TP53 activation also can be observed in pre-cancerous lesions in individuals. Analysis on specimens from urinary bladder, breast, lung, skin and colon carcinoma revealed that pre-carcinogenic lesions are characterized by DDR markers, like the phosphorylation of ATM, CHK2 and H2AX. The effective DDR leads to TP53 activation, indicating a fully active counter-response to the incipient neoplasia, where proliferative markers like Ki67 are completely absent[103,111](Fig.8).

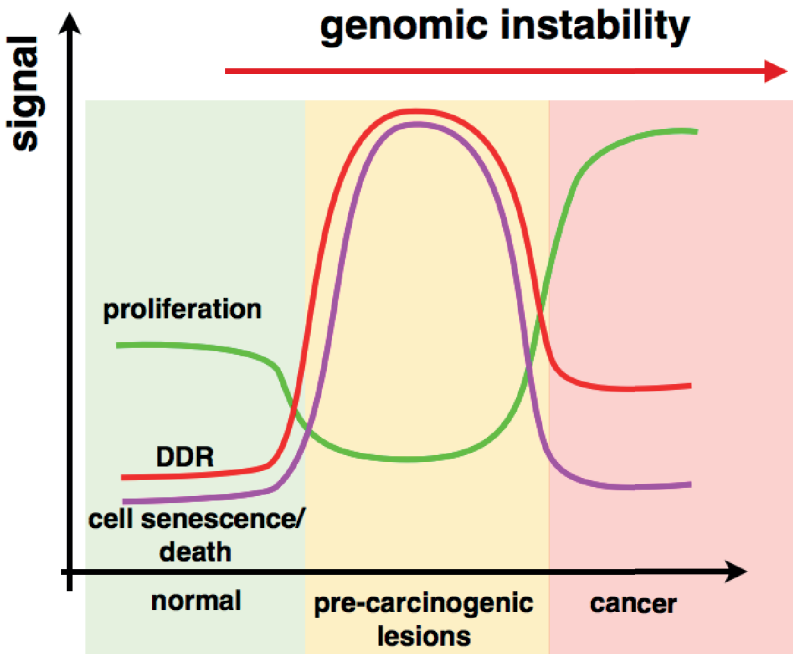


Fig.8: Schematic representation of the tumorigenic barrier. In normal tissues, cell proliferation is stable and there are no signs of damaged DNA and activation of DDR. As genomic instability begins, pre-carcinogenic lesions display an active DDR that blocks cellular proliferation via senescence or apoptosis. In cancer, the DDR that leads to senescence and apoptosis is suboptimal, thus allowing abnormal cellular proliferation.

TP53 is a master regulator of the tumorigenic barrier

The tumor suppressor TP53 plays a key role in activation of the tumorigenic barrier[106]. TP53 is a transcription factors of a multitude of genes[112], including the pro-apoptotic *PUMA*, *NOXA*, *BID* and *BAX*[113]. Following stress signals, like oncogenes activation or exogenous DNA damage, TP53 accumulates in the cellular nucleus, where it facilitates DNA repair and cell-cycle regulation. Nevertheless, in response to overwhelming stimuli, TP53 tips the balance towards senescence or apoptosis, thus preventing cellular transformation[110]. Due to its key role as tumor-suppressor, TP53 is frequently mutated in human cancer[274], and mice that lack one or two copies of the gene are more susceptible to spontaneous and environmentally induced tumors[114-116]. Though TP53 mediates both senescence and apoptosis in normal cells, it remains unclear what determines the choice between these pathways[107], and which response is primarily responsible for tumor prevention.

A. TP53-mediated apoptosis

Numerous line of evidence indicates that apoptosis is an effective regulator of the tumorigenic barrier. The transformation

capacity of *E1A* oncogene in mouse embryo fibroblasts (MEFs) is partially blocked by extensive apoptosis mediated by TP53[117]. MEFs deficient in *BAX,* are rescued from *E1A*-dependent cell death and more easily transformed *in vitro* and *in vivo*[118]. In a mouse model of *c-MYC* driven pancreatic cancer, the oncogene activity in adult β-cells induces tissue proliferation accompanied by overwhelming apoptosis[102]. The apoptosis was mediated by the pro-apoptotic TP53 target gene Bcl-xL. Mice with genetically inactive *BAX* or *PUMA* have similar tumor incidence to TP53$^{-/-}$ genotype[110,118,119].

BAX is the main mediator of TP53-dependent apoptosis.

B.TP53-mediated senescence

The extensive use of cancer cell lines to study cellular biology have brought to the erroneous idea that when DNA damage is not reparable, cells inevitably enters apoptosis. Most studies[120] in primary human fibroblasts show that over-expression of oncogenes like *MYC*, *HRAS*, *BRAF* and *E2F1* leads to a permanent cell-cycle arrest, with all the traits of cellular senescence[101,121-124]. Mice data support the role of senescence as tumorigenic barrier. In a murine model for hepatobiliary carcinoma, genetically restoration of wt-TP53 causes tumor remission via senescence[125]. Senescent hepatocytes seem to trigger their clearance by secreting cytokines that attracts phagocytes. In the human colon, senescent rather than apoptotic cells are found in adenomas, the pre-carcinogenic lesions that precede carcinomas[104].

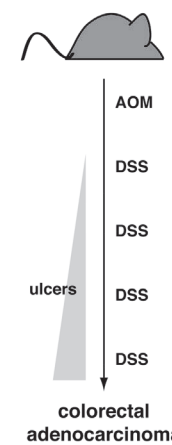
As both cellular senescence or apoptosis have been seen in different tumorigenic studies[100,109,126-128], the activation of either responses seems to be tissue and model dependent, thus both should be assessed in every carcinogenic model.

Genomic instability is not the only enabling hallmark of cancer. To stand DNA damage and survive, pre-malignant cells may need the help of external, pro-survival stimuli. As I will describe in the following paragraph, pro-survival signals can come from an inflammatory response. It is noteworthy that such responses can be triggered by chemicals, genetic predisposition or bacterial infections.

4 INFLAMMATION AND CANCER

Epidemiological evidence indicates that some type of chronic inflammatory syndromes predispose to cancer in humans. Patients suffering from inflammatory bowel disease (IBD) such as Crohn's and ulcerative colitis, are at high risk of intestinal tumors[129], while cirrhosis and hepatitis strongly associate with hepatocellular carcinoma[130].

Chronic inflammation and cancer were proved to be causally linked in animal models and interventional studies. Chronic ulcerative colitis can be induced in mice with a single dose of the genotoxin agent azoxymethane (AOM) followed by repeated administration of the pro-inflammatory chemical dextran sulfate sodium (DSS)[131](Box.5). After 10 weeks, the mouse intestine is severely inflamed with sporadic episodes of colorectal adenocarcinoma (CAC), characterized by classical markers: β-catenin nuclear translocation and c-MYC up-regulation[131,132]. In this model, the small intestine had only minor villi neoplasia, and the colon is confirmed to be the main area for cancer onset of the entire gastrointestinal tract.



BOX.5: Mice model for induction of ulcerative colitis and colorectal adenocarcinoma

A major player in inflammation-driven carcinogenesis is the family of nuclear factors-κB (NF-κB)(Fig.9). NF-κB are transcription factors that assemble in heterodimers and mediate the expression of pro-inflammatory genes[133]. In absence of signals, NF-κBs are prevented from entering the nucleus by inhibitors of NF-κB (IκBs). In response to dangerous stimuli, like cytokines, bacterial and viral products[134], cellular receptor activates the IκBs kinases (IKKs), which phosphorylate IκBs and target them for degradation. Released from their inhibitors, NF-κBs can now enter the nucleus and activate the transcription of pro-inflammatory cytokines like TNF-α, IL-6, IL-23 and COX-2. The IKKs -> NF-κB activation is the canonical pathway of the inflammatory response.

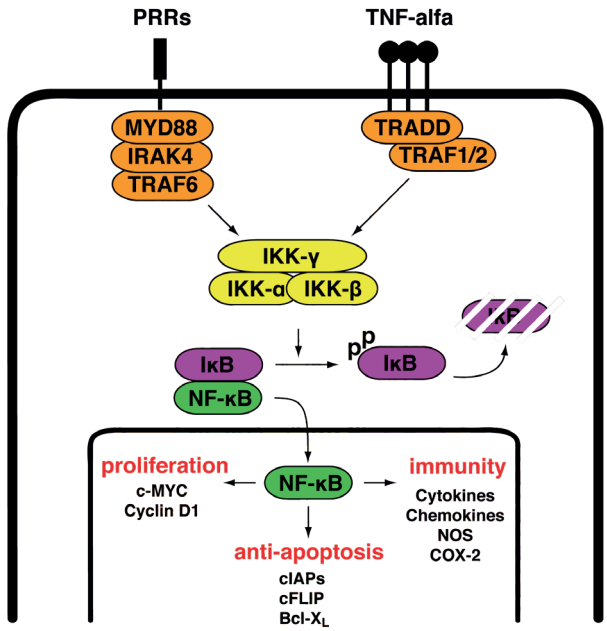


Fig.9: The transcription factor NF-kB is activated via different pathways, mainly TNF- and pathogen mediated

IKK-γ knockout mice die at E.12.5-13 due to sever liver damage caused by apoptosis[275].

There are three different IKKs: IKK-α, IKK-β and IKK-γ. IKK-α and -β are kinases that targets IkBs, while IKK-γ is a regulatory subunit essential for these two kinases activity[135].

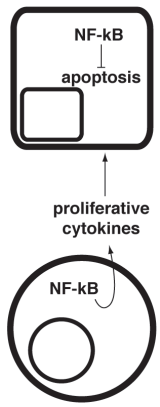
Oncogenes like *CCNDBP1* (Cyclin D1) and *c-MYC* are transcription targets of NF-κB, together with anti-apoptotic Bcl-XL and IAP-1. Due to its pro-survival and proliferation activity, the NF-κB pathway is essential for mouse embryogenesis and wound healing[136].

Relevant for this discussion, bacteria can activate NF-κB signaling via Pattern Recognition Receptors (PRRs). PRRs are specialized sensors of numerous pathogen products that transmit an alert signal to the cells[137]. MyD88 is the adaptor protein that specifically links PRRs to IKKs[138], and dendritic cells depleted for MyD88 fail to activate NF-kB after bacteria exposure[139].

Colorectal cancer

In animal models of ulcerative colitis, the inhibition of NF-κB activity via the use of anti-IKKs drugs[140], or antisense-RNA therapy[141] blocks the secretion of the pro-inflammatory TNF-α, IL-1 and IL-6 in the colon. This inhibition reduces chronic inflammation and CAC development. Other studies indicate that NF-kB exerts a pro-survival function on pre-carcinogenic cells. Mice with cell-specific IKK-β knockout in intestinal epithelial cells

(IECs) (*IKK-β*$^{-/\Delta\ IECs}$) develop less adenomas when put on a CAC regime (AOM + DSS) compare to *IKK-β*$^{-/+IECs}$ litter-mates[132]. In this model, *IKK-β*$^{-/\Delta\ IECs}$ mice had higher IECs apoptosis, though cytokines and inflammation were comparable to controls. When IKK-β was depleted in the myeloid lineage in the same model, proliferative factors like IL-6 and IL-8 were reduced in the colon. These data indicates a dual role of NF-κB in colon cancer: in enterocyte NF-κB plays a pro-survival role, while macrophages of the lamina-propria are responsible for secretion of proliferative factors (Box.6).



BOX.6: In colorectal cancer, NF-kB plays a double role: in epithelial cells, mediate cell survival, while in immune cells is required for the secretion of proliferative factors.

Bacteria can contributes to fuel the pro-inflammatory signaling that precede adenomas. Deficiency in MyD88 (*MyD88*$^{-/-}$) increases the survival of mice genetically predispose to polyposis and adenomas[143]. The reduced number and size of polyps detected was associated with the reduced expression of pro-tumorigenic cytokines like TNF-α, IL-1β and COX-2. The *MyD88*$^{-/-}$ background also prevents adenomas in the AOM+DSS model of ulcerative colitis[143]. In another model of genetically induced CAC with ulcerative colitis (*rag2*$^{-/-}$ x *T-bet*$^{-/-}$, TRUC)[144] the mucus layer in the intestine is thinner compare to controls. This contributed to chronic inflammation, transcription of inflammatory cytokines, ROS production and 8-oxodG DNA damage in intestinal epithelial cells. Finally, the administration of antibiotic reduces the number of tumorigenic and pre-tumorigenic lesions[145], underling the relevance of bacterial-induced inflammation in cancer onset.

Reduced mucus layer is found also in the colon of IBD patients[142].

Certain group of commensal bacteria may exacerbate chronic inflammation. The microbiota composition of TRUC mice was found to be different from the one of matched controls (*rag2*$^{-/-}$)[146]. TRUC colitis could then be transmitted to WT mice following perinatal exposure to a TRUC-mother[144]. Further test on the microbiota composition pointed to the Gram-negative, anaerobic *K. pneumoniae* and *P. mirabilis* as responsible for the transmissible colitis[146]. An ever increasing body of evidence points to the colonization with different commensal bacteria as driving force for life-long diseases, like colitis, allergies, obesity, malnutrition and cancer[147].

H. pylori is a bacterium that colonize the stomach of half of the world population, and some strains increases the risk of

gastritis and stomach cancer[148]. Overnight incubation of *H. pylori* enhances ROS levels and 8-oxoG damage in gastric epithelial cell lines[276]. High ROS levels are also observed in stomach of patients suffering from *H. pylori*-induced gastritis, and antibiotic treatment reduces ROS in the same patients[277]. BigBlue® mice infected for 6 months with *H. pylori* have in average 4-fold increase in gene mutations compare to uninfected mice[149]. As these mutations were mainly transversions, this *H. pylori*-associated genomic instability was attributed to the oxidative stress caused by the host inflammatory cells.

The bacterium *Bacteroides fragilis* is a habitant of the human colon. Enterotoxigenic strains of *B. fragilis* (ETBF) encode for *B. fragilis* toxin (BFT) that causes inflammatory diarrhea in some individuals, while asymptomatically colonize a portion of the human population. In APC^{min} mice, BFT-producing *B. fragilis* triggers colitis and induces more colonic tumors compare to BFT-negative bacterial. The toxin was responsible for the activation of STAT3, a transcription factor that, similarly to NF-kB, mediate immune response. STAT3 was found responsible for the recruitment of pro-inflammatory T helper type 17 (Th17) in the colon, which secretes the pro-inflammatory cytokine IL-17. Antibody blockage of IL-17 inhibits colitis and hyperplasia in the mice. These data indicates the active role of the adaptive immunity in fueling the tumorigenic process[150].

APC is a gene commonly mutated in familial denomatous polyposis (FAP). The APC^{min} mice carry a truncated version of the APC protein and develop up to 100 polyps in small intestine and colon tumors.

Liver cancer

Differently from CAC, the role of NF-κB in development of hepatobiliary carcinoma is less understood. Patients suffering from viral-dependent or -independent hepatitis present higher nuclear localization of NF-κB compare to normal livers[151,152]. This, however, is not conclusive for its role in tumorigenesis. In models where hepatocellular carcinoma (HCC) is triggered by low-grade hepatitis (due to biliary tract disfunction ($mdr2^{-/-}$)[153] or hepatocyte apoptosis (LTα:βhep)[154] in mice) tumors incidence is reduced by genetic impairment of NF-κB activity in hepatocytes[154,155]. In both models, NF-kB prevents apoptosis via transcription of pro-survival genes (A1/Bfl1 and GADD45beta) thus favoring tumor progression. The NF-κB anti-apoptotic activity seems to be essential during late stage of tumor development[155], rather

NF-κB as tumor promoter

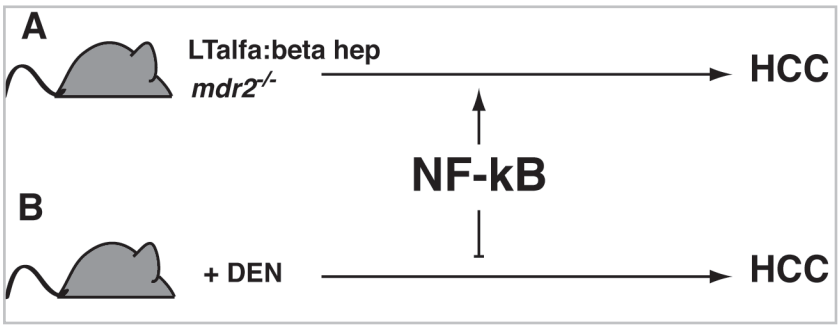


Fig.10: NF-kB can have both pro-tumorigenic and anti-tumorigenic functions in liver. In hepatocytes, NF-kB is a potent anti-apoptotic transcription factor. In some instances, this effect is detrimental because supports survival of pre-malignant cells (A). In other contexts, the survival properties of NF-kB avoid compensatory proliferation, thus preventing expansion of mutated cells that may be tumorigenic (B).

than in the initiation steps [132]. Similarly to the CAC model, the fundamental inflammatory process in *mdr2*$^{-/-}$ mice is maintained after hepatocyte-specific NF-kB inactivation. This points to myeloid cells as the main source of inflammatory cytokines that maintain the inflammatory environment necessary to fuel tumor progression.

NF-κB as tumor suppressor

On the other hand, NF-κB activity is lower in liver metastasis than in primary tumors in patients, favoring a scenario where NF-κB function as tumor suppressor[151]. Consistently, inactivation of NF-κB in IKK-γ knockout adult mice promotes development of spontaneous hepatitis, fibrosis, and HCC[156], and mice deficient for IKK-β in hepatocytes (*IKK-β*$^{-/\Delta hep}$) are more susceptible to HCC after administration of the mutagen diethylnitrosamine (DEN).

How to explain these conflicting data? In the latest models, NF-κB maintains DEN-mutated cells alive and prevents the compensatory proliferation that follows apoptosis, thus limiting the expansion of these cells. This is consistent with the observation that hepatectomy, following DEN-treatment, also promotes HCC via the induction of cell proliferation necessary to regenerate the tissue[157](Fig. ten).

NF-κB can thus function as either promoter or suppressor of tumorigenesis in the liver, and its role in the contest of chronic bacterial infections has not yet been elucidated.

So far, I explained how GI and chronic inflammation favor tumorigenesis. We saw that bacteria can trigger pro-tumorigenic inflammatory response via PRRs and the NF-kB pathway, and favor GI via production of ROS. In the next chapter, I will describe how bacteria can directly cause DNA damage in a ROS-independent manner, focusing on the genotoxin CDT.

5 BACTERIAL PRODUCTS MAY DIRECTLY TRIGGER GENOMIC INSTABILITY

Beside production of ROS, associated with chronic inflammation, some bacterial infections might contribute to induction of genomic instability via alternative mechanisms. Indeed, mouse gastric epithelial cells infected with *H. pylori* shows signs of DNA damage response that could not be prevented by ROS scavengers, or by the combined deletion of genes encoding for nitric oxide synthase (iNOS) and NADPH oxidase 2 (NOX2)[158]. Additionally, *H. pylori* can down-regulate the expression of proteins responsible for the mismatch repair (MMR) pathway *in vitro*[159] and to some extent *in vivo*[160], thus compromising the cellular DDR. Interestingly, the gastric epithelium of patients with stomach cancer shows high-rate of microsatellite instability (MSI)[161], a form of GI that arise from a dysfunctional MMR[162]. *H. pylori* was found more frequently in gastric tumors with MSI compare to tumors without MSI[163]. Today, the protein(s) or the metabolite(s) responsible for the induction of ROS-independent DNA damage or the down-regulation of the MMR proteins remain unknown.

iNOS and ***NOX2*** deficient cells cannot produce ROS following stress stimuli, like cytokines and viral infections.

MSI is the extension or the shortening of repeated sequences in the genome, area particularly sensitive to inefficient mismatch repair. MSI is also seen in colon caner.

Other pathogens, like *Neisseria gonorrhoeae*[278] and *Pseudomonas aeruginosa*[164], have been shown to induce a certain degree of DNA damage *in vitro*, but their mechanism of action remains unclear.

A mechanism for the promotion of GI during chronic bacterial infection may be the production of toxins that directly cause DNA damage. Today, the only characterized bacterial genotoxins are Colibactin and CDTs [1], however their contribution to carcinogenesis has not been fully assessed.

In paper II, we studied whether chronic intoxication with CDT promotes genomic instability. To this end, we have grown different cell lines of epithelial and mesenchymal origin in the presence of sub-lethal doses of the toxin, which did not induce an

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acute cell cycle arrest, but still caused DNA damage as assessed by formation of micronuclei already at 2 days post intoxication. Traits of genomic instability were maintained for one week, and persisted in the sub-lines selected in the presence of the toxin up to 220 days. No detectable changes were seen in cells selected with a mutant inactive toxin at comparable density and passages throughout two independent experiments.

Long-term exposure to sub-lethal CDT-doses leaded to a time dependent increase in mutation frequency of a reporter gene of the immortal rat fibroblasts BigBlue®. Mutations were mainly transversions, while transitions and insertion/deletions were less frequent. This effect was associated with higher frequency in nuclear abnormalities and chromosomal aberrations. Similar effects were also observed after short-term infection of mammalian cells with *E. coli* strains producing Colibactin, the other known bacterial genotoxin[165].

Transversions are exchange of a purine for a purine or a pyrimidine for a pyrimidine, while **transitions** are exchange of a purine for a pyrimidine or a pyrimidine for a purine.

What could promote the proliferation and survival of our cells, in spite of the marked GI induced by CDT? As previously discussed (Chapter 3), one mechanism is the overcoming of the tumorigenic barrier, which can be achieved by impairment of the DDR. Indeed, we could demonstrate that cells selected in the presence of the active toxin showed a significant reduction in sensing the DNA damage, followed by a reduced activation of the downstream checkpoint. Additionally, in our experiment, GI resulted in the production of a heterogeneous population of cells. This favored the insurgence of cells able to survive in absence of anchorage, as we detected more colonies in the soft-agar experiment in cells exposed to the wild type CDT compare to mutant toxin.

MAPKs p38 and cell survival

The activity of MAPKs p38 plays a key role in cell survival following CDT exposure both in acute and chronic intoxication (papers I and II). MAPKs p38 is a group of highly related enzymes encoded by four different genes in human, corresponding to isoform p38-α, -β -γ and -∂. All these isoforms can be phosphorylated in response to stress, like DNA and oxidative damage, hypoxia, ischemia and cytokines[166].

While it is generally accepted that MAPKs p38 function as

tumor-suppressor in normal cells [167,168], in cancer its role is less understood[166]. In paper I and II, and in previous work from our laboratory[47], MAPKs p38 protects cells from apoptosis, thus being pro-tumorigenic. In line with these evidence, epidemiological studies link high levels of phospho-MAPKs p38 with a more malignant phenotype, in lung, thyroid and breast cancer, glioma, follicular lymphomas, and neck squamous cell carcinomas[169-174]. In paper II, it is worth notice that though the relative levels of phospho-MAPKs p38 were higher in these cells, the total amount of MAPKs p38 was actually lower compared to controls. This might suggest a selective pressure for reducing the total amount of this kinase. Other studies have tried to assess the role of MAPKs p38 following DNA damage. Particularly, the ability of ionizing radiation to regulate MAPK p38 activity in cancer cells appears to be highly variable, from no activation[175], to weak and strong activation[176,177]. In lung, head and neck cancer cell lines, an elevated basal level of phospho-MAPKs p38 correlates with resistance to the DNA damaging drug cisplain[178].

We also report that increased level of ROS is another carcinogenic traits of CDT-mediated transformation. High ROS accumulation is a trait of some cancer cells, and might further contribute to overcome the tumorigenic barrier. There seems to be a relationship between MAPKs p38 pathway and endogenous ROS levels in normal and cancer cells. ROS can activate MAPKs p38 in ovarian epithelial tumors[179] and, in turns, MAPKs p38 prevent accumulation of ROS in a breast cancer mice model[180]. Immortalized mouse embryo fibroblasts (MEFs) deficient for MAPK p38-α are more easily transformed by *HRAS* than wild type cells, and this transformation seems to be mediated mostly by the ROS induced by the oncogene[181]. In the early step of transformation, *HRAS* induces reactive oxygen species that can accumulate at high level only when MAPK p38-α is absent. Interestingly, cancer cell lines with endogenous high level of ROS shows sub-optimal MAPK-p38 activation and increased capacity to grown in anchorage-independent manner[181]. Collectively, these data suggest that transformation of immortalized rodent cells like NIH3T3 and BigBlue® is prevented by high levels of MAPKs p38, and its signal must somehow be finely tuned to

allow cellular transformation.

However, other mechanisms can uncouple ROS from MAPKs p38 activation. *GSTM1* and *GSTM2* are members of the GST family, and their over-expression inhibits the oxidative stress-induced activation of MAPKs p38[182,183]. The level of mRNA of these genes increases with the level of ROS in different cancer cells, suggesting that *GSTM1*/2 protect from MAPKs p38-dependent apoptosis caused by oxidative stress.

As I discussed above, the carcinogenic process encompasses numerous mechanisms, and the enabling cancer hallmarks are both GI and pro-carcinogenic inflammation. We have shown in paper II that long-term exposure to sub-lethal doses of CDT is carcinogenic *in vitro*. To fully characterize the molecular mechanisms by which CDTs contribute to tumorigenesis *in vivo*, we focused on the *Salmonella* Typhoid Toxin, and developed a model of chronic infection in immune competent mice. *Salmonella* was chosen as model bacterium since it is the only CDT-like producing pathogen associated with human cancer[184-190].

In the next chapter, I will briefly summarized the mechanisms of *S. enterica* pathogenesis and introduce the model we have developed.

6 *SALMONELLA* BIOLOGY

Salmonella typhi is a facultative anaerobic, Gram-negative bacterium[279] responsible for typhoid fever, an infection disease that causes general ill-feeling, abdominal pain and temperature, due to a systemic bacterial infection. High fever and severe diarrhea might occur during the course of the disease if untreated[191]. Though typhoid fever is rare in the United States and Europe, it is endemic in Asia, Africa and South America[192] and the U.S. Centers for Disease Control estimates at least 22 million people affected worldwide and 200.000 death each year[280].

The correct designation for *Salmonella typhi* is "*Salmonella enterica*, enterica subspecies, serovar Typhi". For simplicity, I will use *S. enterica* to refer to *Salmonella enterica*, enterica subspecies; while *S. typhi* and *S. typhimurium*, respectively, will be use to indicate the two serovars[193-195].

S. enterica pathology

From the prospective of the clinical outcomes caused by *S. enterica* infections, three distinct syndromes have been classified: typhoid fever, septicemia and enteritis. Typhoid fever is a systemic infection in humans that is caused by the *S. enterica* serovars Typhi, Paratyphi A, Paratyphi B, and Paratyphi C. Importantly, each one of these bacteria is a strict human or high-primate pathogen, as none of these serovars are able to infect other mammalians[196].

The characterization of the pathology of *S. typhi* infection in humans comes from pioneering studies in the 1970s where the pathogen was administered to consensus volunteers[197](Fig.11). In one of these largest studies, Hornick *et. al.* infected approximately 200 individuals[191], with doses ranging from 10^4 to 10^9 bacteria. Fever was the first clear outcome, after a median incubation time of 5-9 days, depending on the load administrated. Some individuals manifested constipation at the early stage of infection

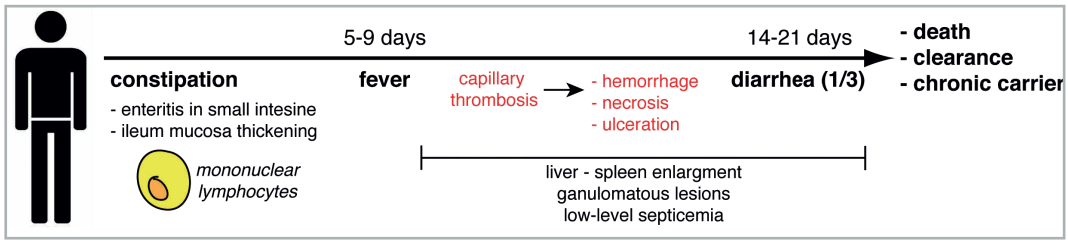


Fig.11: *S. typhi* pathology in humans

and 1/3 developed diarrhea after fever. Additional studies looked at the intestine of volunteers to find *S. typhi* primarily infecting the ileum (the final portion of small intestine), particularly in the Peyer's patches[197,198]. In the ileum, mucosal hypertrophy occurs due to mononuclear cells, with poor polymorphonuclear (PMN) cells infiltration. The fact that PMN cells are not abundantly found at the site of infection, underlines the capacity of *S. typhi* to exert a silent invasion compare to non-typhoidal strains or enterotoxigenic pathogens[199]. Low levels of septicemia are detected in most patients and it is thought to be required for the establishment of a systemic infection[196].

Mononuclear cells that infiltrate inflammatory lesions are mainly macrophages and lymphocytes.
Polymorphonuclear cells are mainly components of the innate immuity, like basophil, neutrophil and eosinophil.

S. enterica infection strategy

As *S. typhi* does not infect mice, *S. typhimurium* became the reference strain to study typhoid fever in rodents, as this pathogen recapitulates many of the characteristic of the human typhoid fever in susceptible mice[196]. *S. enterica* infects the host via ingestion of contaminated food or water. The bacterium survives the low pH level of the stomach[200] and reaches the ileum where it preferentially cross the epithelial layer[196]. *S. enterica* employs flagella to penetrate the mucus, where it resists the numerous natural defenses of the host, such as IgA, digestive enzymes, bile salts and antimicrobial peptides[201-203]. Two different entry routes have been described to cross the epithelium monolayer: active endocytosis via enterocytes[204] and pinocytosis of microfold (M) cells[205] located on Peyer's patches[198]. *S. enterica* is also able to disassemble the cellular tight junctions *in vitro*, thus adding another mechanism to facilitate pathogen penetration across the intestinal epithelium[206].

Although *Salmonella* preferentially invades the host through M-cell pinocytosis, data indicates that the capacity of *Salmonella* to invade and survive within epithelial cells is an essential feature

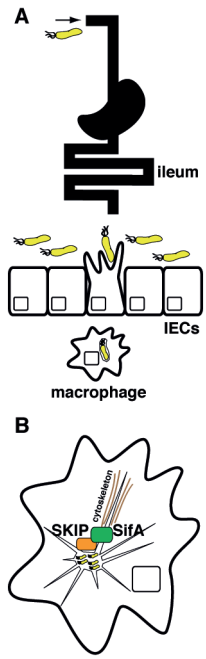
for its pathogenicity *in vivo*[207]. After crossing the intestinal barrier, *Salmonella* is pinocytosed by resident macrophages, where it survives and replicates within the *Salmonella* containing vacuole (SCV)[208,209](Box.7).

To establish a successful infection, bacteria need to subvert the host cellular homeostasis and hijack cellular pathways to their advantage. *S. enterica* does so by injecting effectors directly into the cytoplasm of target cells. To deliver its effectors, *Salmonella* relays mostly on one organelle: the Type III Secretion System (T3SS). T3SS is a needle-like structure composed by a basal core that spans the bacterium periplasm, and an elongated part that injects proteins directly into the eukaryotic cell[210]. Secretion Systems are common among pathogenic bacteria, and are usually encoded in genetic island together with their secreted effectors. *Salmonella* encodes for two T3SS in distinct pathogenicity islands: SPI-1 and SPI-2. There are about 40 virulence factors secretesd via SPI-1/2 T3SS characterized up to date[207] and more then 300 have been predicted[211]. Both SPIs are essential for *Salmonella* pathogenicity and host colonization, but they exert different functions.

SPI1-T3SS acts at the interface between the bacterium and cell and delivers its effectors through the plasma membrane. SPI1 is responsible for bacterial uptake into non-professional phagocytic cells, such as epithelial cells, to cause intestine inflammatory response, diarrhea and colonization. SPI-2-T3SS acts inside the SCV compartment, where it promotes survival and the establishment of a systemic infection[207].

Inside the SCV, *Salmonella* changes the expression profile of more than 900 genes[208], which help the bacterium to survive in this hostile compartment. SCV stimuli trigger the change in the LPS structure of the bacterium[212], the modification of the peptidoglycans wall[213], and the down-regulation of flagellin and SPI-1[214].

Of all SPI-2 effectors, SifA is essential for the maturation and integrity of this vacuolar compartment (Box.7b). Mutants for *SifA* show a cytoplasmic distribution, reduced intracellular replication and virulence[215]. SifA is injected to the outer-leaflet of the SCV, from where it mediates the formation of *Salmonella*-induced



BOX.7: (A) *Salmonella* enters the host via contaminated food or water. At the level of the ileum, it crosses the epithelial layer via M-cells and it is uptake by resident macrophages. (B) Inside cells, *Salmonella* replicates within a vacuole, which homeostasis depends on SifA and SKIP proteins.

filaments (Sifs)[216]. Sifs are protuberances that grow along the microtubular cytoskeleton[217,218]. To produce Sifs, SifA binds to the host protein SKIP (SifA kinesin interacting protein) that connects the SCV to the microtubule motor kinesin. Additionally, the SifA carboxyl-terminus domain recruits members of the RHO small GTPase family to initiate endosomal tubulation[219].

Chronic *S. typhi* infection

A significant percentage (1–6%) of people infected with *S. typhi* become chronic, asymptomatic carriers[220]. Since no other natural niches are known for *S. typhi*, chronic carriers serve as *Salmonella* natural reservoir, from whom the bacteria are periodically shed through feces[221]. In humans, *S. typhi* can establish an acute, active infection of the gallbladder, accompanied by inflammation (cholecystitis). The gallbladder is a small organelle of the liver, located above the duodenum, which aids fat digestion via the release of bile salts. *S. typhi* has been found in the gallbladder long after the end of the typhoid symptoms in patients, suggesting unique mechanisms used by *S. typhi* to mediate a long-term colonization in a hostile, bile-rich environment[222].

The development of a chronic typhoid infection is frequently associated with the presence of gallbladder abnormalities, especially gallstones[223]. In typhoid endemic area, 5% of patients enrolled for gallbladder removal carry *S. typhi* as biofilm on gallstones[224]. In animal models, gallstones were shown to facilitate the formation of *S. enterica* biofilm, increasing colonization of the gallbladder and enhancing fecal shedding[224]. Although subjects who carry *S. typhi* often have biliary-tract disease, this condition is not an absolute requirement for the development of a carrier state[220,225,226]. In patients carrying both *S. typhi* and cholesterol gallstones in the gallbladder, antibiotics are typically ineffective against the bacterium[223] and their risk for developing hepatobiliary carcinomas is higher than acutely infected patients [187,188].

7 A NEW *SALMONELLA* INFECTION MODEL

Today, no model that recapitulates *Salmonella* long-term infection and its role in cancer development has been established. Furthermore, the role of Typhoid Toxin in this process still remains to be elucidated. Here I present preliminary and unpublished data on a new animal model that we designed to address these issues.

Mice models of *S. enterica* infection

S. typhimurium is the standard pathogen to study typhoid infection in rodents. The classical animal model used for *S. typhimurium* infection and virulence tests are BALB/c and C57BL/6 mice (Fig.12). These strains are exquisitely sensitive to infection, and as little as 10 bacteria can cause death[227]. Signs of disease (i.e. elevated temperature) appear between 4–8 days after oral inoculation, however, diarrhea does not develop. Intestinal inspections reveal enlarged Peyer's patches and a thickening of the ileum mucosa. Diffuse enteritis is present in the small intestine characterized by a predominantly mononuclear infiltration[228]. Hyperplasia of the follicular area in the lymphoid tissue, capillary thrombosis, hemorrhage, and ulcerations may be present in the terminal ileum, while other areas of the intestine remain largely intact. The intestinal pathology and inflammatory reaction in mice is hence similar to that of typhoid fever patients[197].

S. Typhimurium in mice leads to a systemic bacterial dissemination. The rapid bacterial multiplication in the liver and spleen results in the hepatomegaly and splenomegaly[229]. Growth of bacteria in the mesenteric lymphnodes, the liver and the spleen triggers the formation of abscesses containing predominantly neutrophils. These microscopic lesions become enlarged and transform into granulomas, with central necrosis and peripheral mononuclear leukocytes[230]. In these lesions, *Salmonella* resides

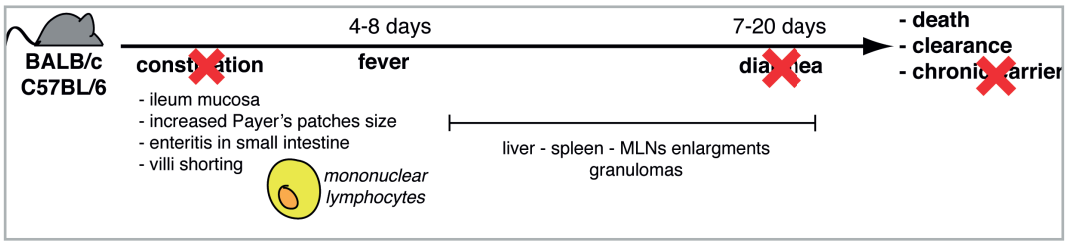


Fig.12: S. typhimurium pathology in susceptible mice, which is similar to that induced by *S. typhi* in humans, without constipation, diarrhea, and establishment of a chronic carrier (indicated by the red crosses)

within macrophages[231]. The granuloma observed in mice are similar to those present in tissues of chimpanzees infected with serovars Typhi[232].

Contrary to BALB/c, the sv129 mouse strain is known to be resistant to *S. typhimurium*. This is due to the presence of a wild type version of the gene encoding for the natural-resistance-associated macrophage protein 1 (*NRAMP1*), found inactive in both BALB/c and C57BL/6[233]. NRAMP1 is a macrophage-specific ion pump of the SCV, which restricts bacterial replication[233-235]. Monack *et. al.* showed that the sv129 mice can be infected with *S. typhimurium*, and that 10^8 bacteria per mouse still does not reach the LD50[234]. Despite the absence of fever, the animals bear *S. typhimurium* in mesenteric lymphnodes up to one year, and sporadically shed bacteria through feces.

MC71 is a *S. typhimurium* strain that carries a mutation within the phosphorylase (PNPase) gene, a general regulator of virulence. MC71 shows a reduced virulence *in vivo*, and can establish long-term infection in BALB/c mice[236]

Based on these data, we choose as animal model the sv129 mice, and as bacterium the S. typhimurium strain MC71. Since *S. typhimurium* does not express the Typhoid Toxin, we cloned the TT operons into the chromosome of the MC71 strain by site directed recombination (MC71-CDT). Each subunit of the toxin was tagged with a different epitope for detection by Western-blot or fluorescence microscopy. As control, we used an isogenic strain that lacks *cdtB* (MC71-ΔcdtB).

PRELIMINARY DATA

The experimental set up consisted of three groups of mice: one group was left uninfected, one group was infected with the control MC71-*ΔcdtB* strain, and one was infected with the strain expressing the active genotoxin (MC71-CDT) (Fig.13). Eight mice per group were sacrificed after 30 days, 60 days and 180 days post-infection (p.i.), and we collected the intestine, mesenteric

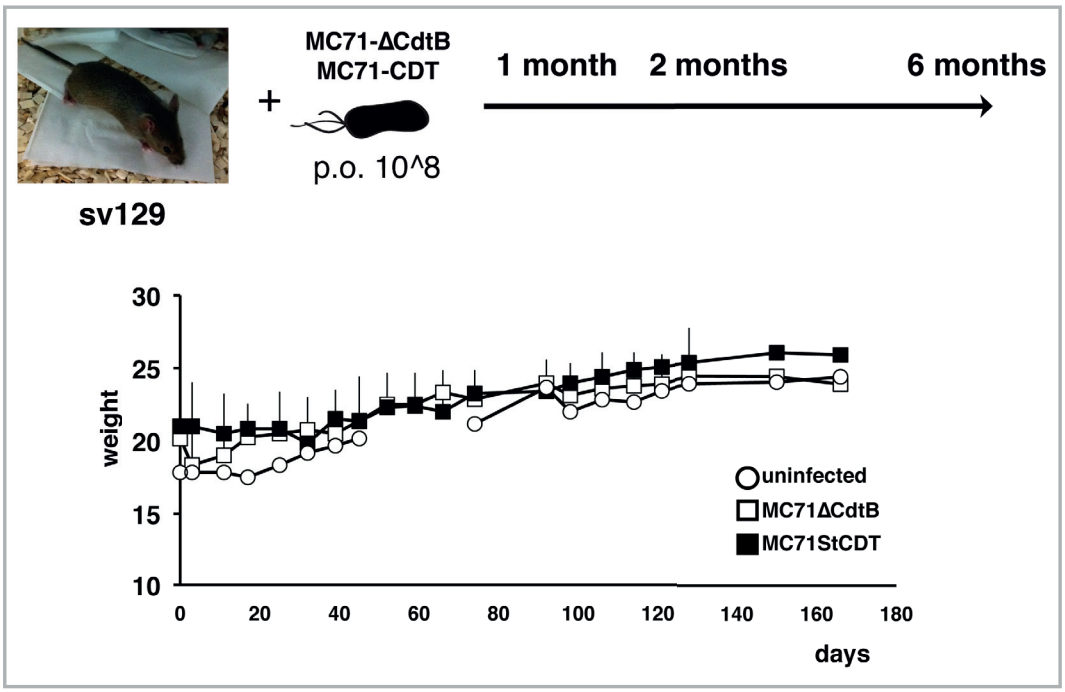


Fig.13: Schematic representation of sv129 chronic infection with MC71 strains. Mice were infected orally (p.o.) with 10^8 MC71-ΔCdtB and MC71-CDT, for one, two and six months. Lower panel: animal weights in grams.

lymphnodes, liver and spleen to assess the bacterial load, while part of each tissue was preserved for histological examination and RNA extraction. Stools were also collected at the indicated time points for analysis of the intestinal microbiota.

Successful infection was determined by recovery of bacteria from the mesenteric lymphnodes, liver and spleen of all the infected mice 30 days and 60 days p.i., although the levels of bacteria recovery decreased of approximately 10 to 100 folds at 60 day p.i.. *Salmonella* was found only in the mesenteric lymphnodes in 40% of the mice 6 months p.i.. Differently from previous study[234], we could not recover any bacterium from the gallbladder and this discrepancy may be due to the different bacterial strain used.

We found no differences in the infection burden of *S. typhimurium* between mice infected with the MC71-CDT and MC71-*ΔcdtB*, indicating that the presence of the toxin does not influence colonization in this model. Other studies, however, have shown that CDT in *H. hepaticus* and *C. jejuni* seems to

facilitate colonization at 4 months p.i. in immune competent mice[237,238]. It is conceivable that this discrepancy is due to the different type of disease induced by these bacteria. *H. hepaticus* and *C. jejuni* cause a strong inflammatory response but do not spread systemically, while *S. typhimurium* causes a mild enteritis, followed by a rapid systemic infection.

From a histological point of view, we observed an enhanced inflammation of the liver in mice infected for 60 days, with the MC71-CDT strain compared to mice infected with the control MC71-*ΔcdtB* or uninfected mice. At 180 days p.i., the inflammation in the liver subsided, but we could observed a strong lymphatic hyperplasia in 4 out of 5 mice infected with the strain carrying the active genotoxin while this was not detected in any of the mice infected with the control strain.
These data suggest that, although 180 days is not a sufficiently long period to detect any sign of pre-canceous/cancerous lesions in immunocompetent animals, the presence of typhoid toxin enhances the host pro-inflammatory response. This is supported by the preliminary data of the microbiota analysis, performed by High Taxonomy Fingerprint microbiota array[239] on the stool collected 7, 30 and 60 days p.i., which detected an enrichment of bacteria, such as *Fusobacteriaceae*, *Bacteriaceae* and *Lactobacillaceae*, associated with pro-inflammatory/pro-carcinogenic environment[240]. We are currently analyzing the mRNA profile of the organs in infected and non infected mice 60 days p.i. using GeneChip microarray, focusing on genes involved in DNA damage and host immune responses. We will further analyze the extent of oxidative damage in the relevant tissues.

The data, while promising, indicate that we would need to switch to a mouse model which is already prone to develop tumor. This will possibly allow us to consistently reduce the infection period. To address this issue, we have infected mice deficient for the tumor suppressor gene TAp73[241]. together with matching controls. Both strains have functional *NRAMP1*. The experiment is currently on going while writing this thesis.

To demonstrate the importance of the choice of the model, the carcinogenic role of Colibactin in the development of colorectal cancer was recently demonstrated in germ free IL-10 knockout mice treated with the carcinogen azoxymethane (AOM)[242]. Mice mono-colonized with *E. coli* expressing Colibactin have higher incidence of adenocarcinoma than mice mono-colonised with a Colibactin-deficient *E. coli*[242].

8 SECRETION OF TYPHOID TOXIN

Several interesting questions related to the Typhoid toxin remain to be elucidated. It is known that the toxin expression is induced once the bacterium is internalized and replicate inside the SCV of the infected cell[12]. However in order to reach the nucleus, the toxins has to be secreted from the producing bacterium and cross several cellular membranes. Therefore in paper III we have addressed these issues. For this study we used the *S. typhimurium* strain developed for the *in vivo* model described in the previous chapter. This bacterium has the additional advantage to be a lower risk pathogen compare to *S. typhi*, which matched our laboratory facility. We confirmed that the expression of all three toxin genes is induced in MC71-CDT in conditions that mimic the SCV *in vitro*, validating the model.

Spano *et. al.* have previously shown that secretion of the toxin from the bacterium does not require the *Salmonella* SPI-2 T3SS[243], however all the there subunits present a signal sequence that direct their secretion into the periplasmic space[244]. Based on this evidence, we investigated whether the Typhoid Toxin was released from this location via outer-membrane vesicles (OMVs).

OMVs are spherical lipid bilayers that bud off the outer-membrane (OM) of Gram-negative bacteria. OMVs have been found to be released also from Gram-positive bacteria[280]. Vesicles dimension span between 20 to 250 nm, and mass-spectrometry analysis indicates that OMVs are composed primarily by OM lipids and periplasmic proteins, ruling against bacterial lysis as responsible for their formation[245]. Moreover, OMVs generation does not result from a random budding of a bacterium surface, as only a selected portion of periplasmic proteins and LPS are found in the vesicles[246,247]. Ample evidence demonstrate that bacterial toxins, such as leukotoxin from *Actionobacillus*[246], the heat-labile toxin from enterotoxigenic *E.coli*[247] and anti-bacterial components released by *Pseudomionas*[248], can be found in OMVs.

In paper III, we demonstrated that also TT is secreted within OMVs in conditions that mimic the SCV (*in vitro*) and in infected cells. Fluorescent and electron microscopy allowed the detection of OMVs within the SCV and in the cellular cytoplasm. Similar to our fundings, previous studies have demonstrated the presence of OMVs in the cytoplasm of *Salmonella*-infected cells[249], and the PagK-family proteins were also reported to use OMVs to translocate into the cytoplasm of macrophages[250].

PAPAER III

PagK is a family of small proteins important for *Salmonella* virulence

Differential centrifugation of the culture medium confirmed that the toxin-loaded OMVs were released into the extracellular environment, and this process required an intact SCV, microtubule and actin cytoskeleton, indicating an active transport of OMVs toward the plasma membrane. Bacteria that are unable to form an intact vacuole, such as SifA mutants, did not secreted toxin-loaded OMVs in the cellular medium. This is in agreement with previous data showing that SifA and its host interacting partner SKIP may promote the fission of vesicles from the SCV and their kinesin-1-dependent anterograde transport[251]. The importance of SifA and the formation of Sifs for *Salmonella* replication and virulence have been demonstrated in different models[252], however up to date there are no factors secreted by *Salmonella* infected cells documented to use kinesin-1–dependent anterograde transport.

SKIP: "SifA and kinesin interacting protein"

Once the toxin is released in OMVs from the infected cells, it has to be internalized by bystander cells to exert its genotoxic activity. We used dynamin-1 inhibitor to demonstrate active internalization of OMVs. Once internalized, the TT required an intact Golgi complex to exert intoxication, pointing to the OMVs retrotranslocation as possible trafficking pathway. A summary of these founding is depicted in Figure 14.

The mechanisms by which OMVs deliver their content to cells were found to be different depending on the model bacterium. OMVs produced by *A. actinomycetemcomitans* fuse with cholesterol rich domains of the target cell membrane and release their content into the cytosol[253]. Conversely, Enterotoxigenic *E. coli* (ETEC) OMVs expose on the surface the heat-labile enterotoxin (LT), which mediates binding to the GM1 receptor in

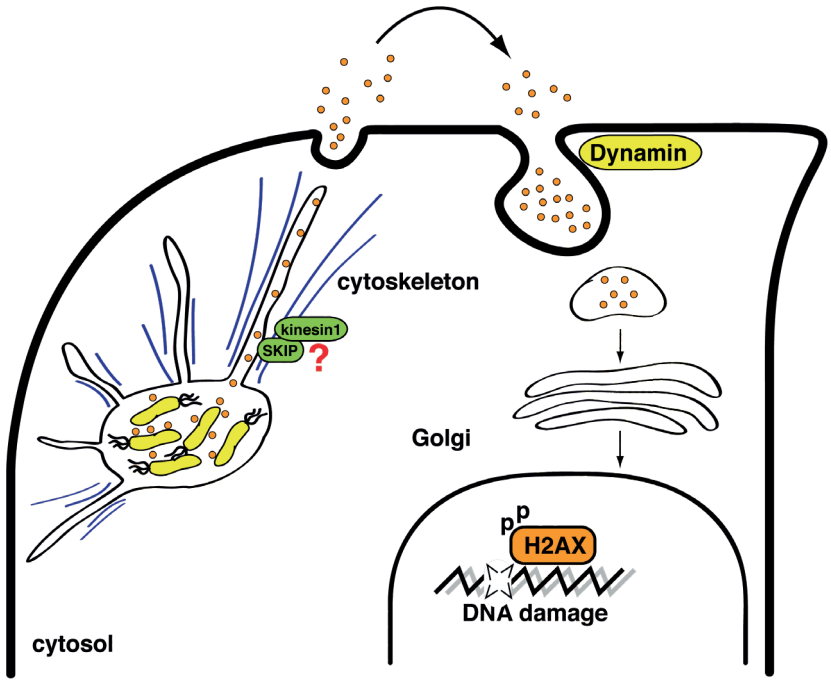


Fig.1 4: *Salmonella* replicates within the SCV in the cytosol, from where it exploit the actin cytoskeleton to secret OMVs to the extracellular environment. OMVs are further uptake in cells following the endocytosis pathway.

caveolin-positive cholesterol domains[247] and are endocytosed in a clathrin-independent manner[254]. *H. pylori* OMVs that carry the VacA toxin do not require lipid rafts for their internalization, while intoxication is partially clathrin-dependent[255].

Interestingly, the presence of bacterial toxins seems to enhance OMVs binding on cell membrane for ETEC and *H. pylori*. Though we did not perform a comparative analysis in our study, it is unlikely for TT to mediate OMVs binding as the toxin is hidden inside vesicles, differently from LT and VacA[256].

Why OMVs?

The advantages for a bacterium to secret its effectors and toxins via OMVs are numerous, since this allow i) to deliver insoluble material; ii) to protect material from antibodies and proteinases; iii) to deliver high-concentration of the product.

Secretion of insoluble material

The *P. aeruginosa* quorum sensing proteins PQS[257] are a group of highly hydrophobic peptides. PQS contributes to the resistance to UV irradiation, and synchronizes the entry of the bacterial population into the stationary phase[258]. PQS have be found to regulate their own package into secreting vesicles[259].

Adhesins are transmembrane proteins that mediate cell-to-cell bacteria aggregation and facilitate colonization in tissue as biofilm formation. *Porphyromonas gingivalis* produces OMVs with adhesins, and these vesicles can induce cellular aggregation and aid the development of dental plaques[260,261].

Protection from the environment

Soluble proteins are exposed to a variety of proteases and digestive enzymes present in niches colonized by the bacteria. Delivery of sensitive-effectors inside vesicles confers protection and may allow them to reach distant targets[262,263]. In our study, TT was found protected from trypsin digestion and antibody detection in OMVs secreted from infected cells.

High-concentration of delivered product

OMVs can co-transport different molecules at the same time, increasing the delivery efficacy and favoring a synergic action. OMVs secreted by the Gram-positive bacteria *Bacillus anthracis* contain a ratio of 10:1 of anthrax toxin components protective factor (PA): lethal factor (LF)[264].

OMVs in pathogenesis

Regardless their study *in vitro*, little evidence has been produced so far to indicate that OMVs are important mediator of bacterial pathogenesis.The primary reason is the lack of knowledge on the mechanisms that regulate OMVs shedding and the consequent inability to produce bacterial strains that lacks vesicles production. *H. pylori* can release OMVs following epithelial cell infection *in vitro*, and EM analysis found vesicles-like structure in patients infected with *H. pylori*, indicating that they are produced *in vivo*[265]. The presence of OM proteins in the serum of rat during overwhelming *E. coli* infections might point to a possible role of OMVs during septic shock [266], and OMVs secreted by *Borrelia burgdorferi* were detected in the serum of human and dogs clinical samples as well as in experimentally infected mice[267].

In the context of bacteria-associated carcinogenesis, TT-loaded OMVs may have a double effect on the carcinogenic potential of *Salmonella* infection: i) efficient delivery of a genotoxic agent that causes DNA damage and may promote genomic instability, as shown for soluble CDT (paper II); ii) chronic activation of non

classical oncogene, such as NF-kB and STAT3[268], mediated by the LPS and possibly other pathogen products may promote the survival of cells upon the genotoxic-dependent carcinogen hit.

Conclusions and future prospectives

This thesis aimed to dissect the molecular mechanisms underling bacterial-induced carcinogenesis. I presented three works where we describe different aspects of the bacterial genotoxin known as Cytolethal Distending Toxin (CDT). Specifically, i) we identified a novel survival pathways triggered by acute CDT intoxication (paper I); ii) we described the molecular changes in response to chronic CDTs exposure *in vitro* (paper II) and set up of a new animal model to assess the carcinogenic potential of CDT-like-producing bacteria; and iii) we characterized a novel exocytosis-like process for paracrine delivery to the target cells of genotoxin-loaded vesicles produced by intracellular bacteria (paper III).

Numerous questions remain unanswered. The NET1-RHOA survival pathway triggered by cancer cells to delay/avoid apoptosis was partially expanded by the discovery that the FEN1 is an important component in the activation of RHOA. It remains to elucidate whether FEN1 affects NET1 translocation to the cytoplasm, or activates RHOA in a NET1-independent manner. To accomplish this, we could deplete FEN1 with iRNA and look for NET1 translocation after DNA DSBs. Alternatively, we could express a tagged-NET1 in cells, and immuno-precipitate this protein before and after DNA damage to test whether it co-precipitates with FEN1, thus suggesting a direct regulatory effect. Another important step would be to identify whether the catalytic activity of FEN1 is important for RHOA activation. To this end, we could expose FEN1-inactive MEFs to CDT[269] and assess whether this would alter RHOA activation compare to MEFs expressing a functional protein. Ultimately, does NET1-RHOA play a role in cell survival of normal tissue, thus affecting carcinogenesis *in vivo*? Cell survival pathways can be significantly different between normal and cancer cells, as shown for the activity of

MAPKs p38 . Animal models where the dominant negative, or the constitutively active form of RHOA is placed under the control of a tetracyclin-inducible promoter may be instrumental to answer this question.

In the contest of cancer therapy, where genotoxic agents are delivered to cancer cells for a prolonged period of time, the study in paper II clarifies that cell lines can acquire a precise set of tumorigenic traits, if the DNA damage is not sufficient to trigger apoptosis. A recent article used genome sequencing data of acute myeloid leukemia before and after chemotherapy, and tracked the genetic changes of the relapsed disease[270]. It would be interesting to conduct a similar analysis on other solid tumors before and after the therapy, and specifically investigate whether genes that promote ROS, increase MAPKs p38 activity and alter the DDR would be found, thus proving these pathways as important players *in vivo*.

Our animal model will contribute to elucidate the function of typhoid toxin during chronic infections with typhoid strains. We would like to study where and when the toxin is actually produced *in vivo*, whether the toxin affects the genomic instability and the cancer progression during bacterial infection, and whether CDT could influence the immune response elicit against the pathogen.

I find particularly interesting the ability of *Salmonella* to reside inside macrophages and avoid killing. Most studies have looked at the mechanisms that this pathogen has developed to avoid lysosomal degradation or to quench nitric oxide within the SCV. In paper III, we found that *Salmonella* secretes vesicles to the extracellular environment via an exocytosis-like process. In the contest of an *in vivo* infection, where engulfed macrophages are surrounded by other cells, toxin secretion might prolong survival, by killing approaching effectors cells, like T lymphocytes. The use of *in vitro* models where infected macrophages are co-coltured with different subsets of T lymphocytes can be used to asses whether kinesin-1 secretion of components, and specifically TT secretion, promotes survival within macrophages via selective killing of the adaptive immunity effector cells.

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I don't have it, but I have a blog.
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