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Epigenetic modifications abolish the expression of the long pentraxin PTX3 in human tumors

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Summary

PTX3 is a fluid-phase pattern recognition receptor that participates in innate immunity and inflammation by modulating complement activation, leukocyte recruitment, extracellular matrix deposition and angiogenesis. PTX3 is a biomarker of inflammatory conditions in different pathologies in humans, including acute myocardial infarction to autoimmune diseases, infections and cancer associated inflammation. Moreover, in *vivo* studies indicate that PTX3 is involved in cancer development, possibly by regulating inflammation. Several tumors lack PTX3 expression, such as human esophageal squamous cell carcinoma, where PTX3 is silenced through promoter hypermethylation. Taken together, these data suggest that PTX3 is potentially involved in cancer development.

The aim of this study was to identify the regulatory elements involved in the modulation of PTX3 expression and gain insight into their mechanisms of action in basal condition, inflammatory responses and cancer.

Using in silico analysis of PTX3 gene we identified two putative PTX3 enhancers located 230 kbp upstream of the promoter and in the second exon of the gene, overlapping a CpG island, respectively. We performed ChIP assay for histone modifications and epigenetic complexes for the analysis of these genetic elements in human cell lines, before and after treatment with TNFα, that induces PTX3 expression. The results show that these regions are enhancers, but in basal condition are enriched with repressive markers H3K27me3 and polycomb group subunities (SUZ12 and EZH2). After TNFα treatment, the two enhancers became active gaining H3K27ac and the RNA Polymerase II and the first enhancer also acquired the binding for NF-kB.

We also analysed the effect of the microRNAs, which were predicted to directly target *PTX3*, using bioinformatics analysis. miR-9, miR-29 family and miR-181 family were shown to directly target the PTX3 3'-UTR and to significantly reduce both PTX3 mRNA expression and protein production. Luciferase assay with PTX3 promoter and with a reporter vector for NF-kb showed

that these miRNAs are also involved in signalling pathways controlling PTX3 transcription. Moreover, RNA Immuno Precipitation assay demonstrated that miR-9, miR-29 family and miR-181 family members and PTX3 mRNA were enriched in the RISC complex of macrophages after 6h of stimulation with lipopolysaccharide. Moreover, inflammatory miRNAs, such as miR-146a, miR-155 and miR-132 regulate PTX3 messenger and protein expression, as well, targeting the network upstream of PTX3 expression. This suggests that PTX3 gene is strictly regulated by several miRNAs acting at different points during inflammatory response.

Bioinformatics analysis showed that PTX3 is not express in several cancer, including colorectal cancer (CRC). ELISA and gene expression analysis in CRC cells confirmed bioinformatics data and the treatment with the demethylating agent 5'aza-dC restored PTX3 expression and production. ChIP assay for histone modifications and transcription factors in CRC cells showed that PTX3 enhancers were inactive and they became active after treatment with 5'aza-dC and in response to inflammatory stimuli, thus acquiring the RNA polymerase II and NF-kB.

Finally, by Methylated-CpG Island Recovery Assay we analysed the methylation of both promoter and enhancers of PTX3 in different CRC cells and in 40 CRC patients of different tumor stages. Data demonstrated that PTX3 regulatory regions were significantly methylated in all the cell lines and in all tumour stages, compared to their healthy counterparts. Moreover, the methylation started from the adenoma. Taken together, these data show that PTX3 methylation is involved in the inhibition of the PTX3 expression in CRC and suggest that PTX3 may have a protective role in the pathogenesis of this tumour.

Introduction

1. Epigenetic regulation

In eukaryotic cells, gene regulation is controlled for the whole cell cycle life, by a complex molecular network of interactions made by several players, like transcription factors, RNA polymerases, regulatory sequences, and co-regulator proteins. Epigenetics is an essential regulator of this interplay. The term epigenetics was introduced for the first time in the nineteenth century by Conrad Waddington, who defined it as ‘‘the branch of biology which studies the causal interactions between genes and their products which bring the phenotype into being.’’[1]. Today the current definition of epigenetics is the set of mechanisms that regulates gene expression without entailing modifications to the DNA sequence, but that are meiotically and/or mitotically heritable [2, 3, 4]. In this great and complicate network, the final effects are the sum of the interactions of each single modification that can lead a positive or a negative regulation. Gene expression is epigenetically regulated through the modulation of chromatin structure and DNA and RNA-based biological processes, like the binding of transcription factors to regulatory regions, such as promoters and transcription elongation. Epigenetic modifications are fundamental for the mobility of transposable element, DNA-protein interactions, gene and microRNA expression, cellular differentiation, embryogenesis, inactivation of chromosome X and genomic imprinting [5]. Each single cell has the own epigenetic mark, which characterizes the state, age and type of cell. Alterations of epigenetic mechanisms, like a modification added in a wrong place or in a wrong time, induce the development of many diseases [6]. This is a further evidence of the major role of epigenetics in maintaining normal development. Many epigenetic marks, like DNA methylation and microRNAs silencing, are highly adaptable, in order to respond to both external and internal

factors. Epigenetics, which allows the interaction between genome and external environment, is involved in pathogenesis of diseases. Indeed, various disease-associated nutritional, chemical, physical and psychosocial factors have been correlated with changes to the epigenome, particularly DNA methylation [7]. Furthermore epigenomic variations can regulate both deterministic and stochastic choice of allelic expression, which correlate to complex pathologies in human populations.

1.1 Epigenetic mechanisms

Epigenetic mechanisms work together creating a complex network of interactions, an ‘‘epigenetic landscape’’, for the control of gene expression at both transcriptional and translational levels.

Based on the type of action, it is possible to distinguish four main groups of epigenetics modifications: nucleosome positioning, covalent histone modifications, DNA methylation and miRNAs regulation.

1.1.1 Chromatin and nucleosome positioning

Chromatin is a complex of macromolecules consisting of DNA packaged by histone proteins. This structure allows to DNA to be compacted in the cell, prevents DNA damage, reinforces the DNA macromolecule during mitosis and regulates gene expression and DNA replication.

Chromatin is organized in three levels. The principal unit of chromatin is nucleosome, which is made by 146 base pairs of DNA rolled up around an octamer of histone proteins, composed by a tetramer of 2 histone 2B (H2B) and 2 histone 2A (H2A) molecules, flanked by dimers of H3 and H4 histones [8]. This first level of organization is know as euchromatin. Nucleosomes are bound by histone linkers (H1 or H5) with consequent further compaction into 30 nm fibres,

which represent the second level of chromatin organization, the heterochromatin. Finally, during mitosis and meiosis the 30 nm fibers are further packed (Figure 1).

The location of nucleosome histone octamer, the nucleosome positioning, depends on the curvature and rigidity of the DNA sequence. This location is a key mechanism for several biological processes, like gene transcription. The presence of the nucleosome acts like a transcriptional repressor, impeding the binding of proteins, as polimerase II and transcription factors, to DNA. Indeed a transcriptionally active gene is characterized by a nucleosome-depleted region (NDR) directly upstream of the promoter transcription start site (TSS). On the contrary, the nucleosome covers the TSS of transcriptionally inactive genes, impeding the binding of transcription activators [8]. Nucleosome occupancy is regulated by ATP-dependent chromatin remodeling enzymes [9, 10], which are multi-protein complexes that alter the nucleosome structure through the use of the energy derived from ATP hydrolysis. ATP dependent chromatin remodelers are classified in base of their mechanism of action. Some complexes bind nucleosomes and adjacent DNA linker and hydrolyze ATP to slide nucleosomes in the direction of the linker, while others can twist nucleosomes or create a loop with another DNA region (Figure 2). These remodelers can both activate and repress gene expression by modifying nucleosome occupancy pattern [11, 12]. In-vitro experiments showed that these enzymes maintain an equal spacing between nucleosomes, organize the nucleosome structure and also an higher-order chromatin structures. Moreover some remodelers are involved in the crucial steps of cell growth and division, like in DNA double-strand break repair, regulate transcriptional repression, progression of cell cycle and are important for the maintenance of embryonic stem cells pluripotency [13]. Mutations or alterations of this enzyme activity are found in some type of cancers, favoring self-sufficiency in cell growth [14].

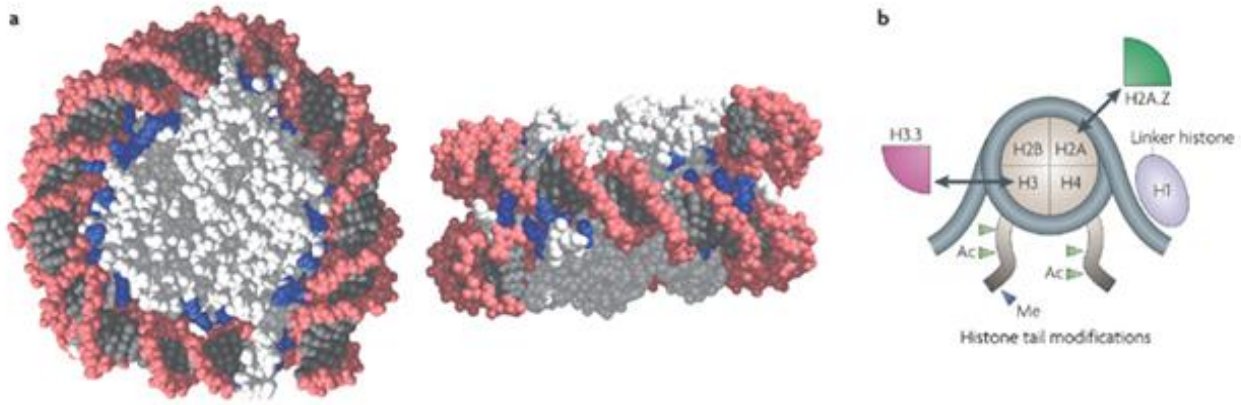


Figure and legend are from: Cizhong Jiang & B. Franklin Pugh *Nucleosome positioning and gene regulation: advances through genomics, Nature Reviews Genetics* ,2009

Figure 1: Structure of a nucleosome core.

Histones are shown in light grey, and the DNA helix is shown in dark grey with a pink backbone. Basic amino acids (lysine and arginine) within 7 Å of the DNA are shown in blue to emphasize the electrostatic contacts between the DNA phosphates and the histones. **b** | A schematic of DNA wrapped around a nucleosome. Examples of histone tail modifications (Ac, acetylation; Me, methylation) and histone variants (H2A.Z and H3.3) are shown. Arrows indicate the replacement of canonical histones with histone variants

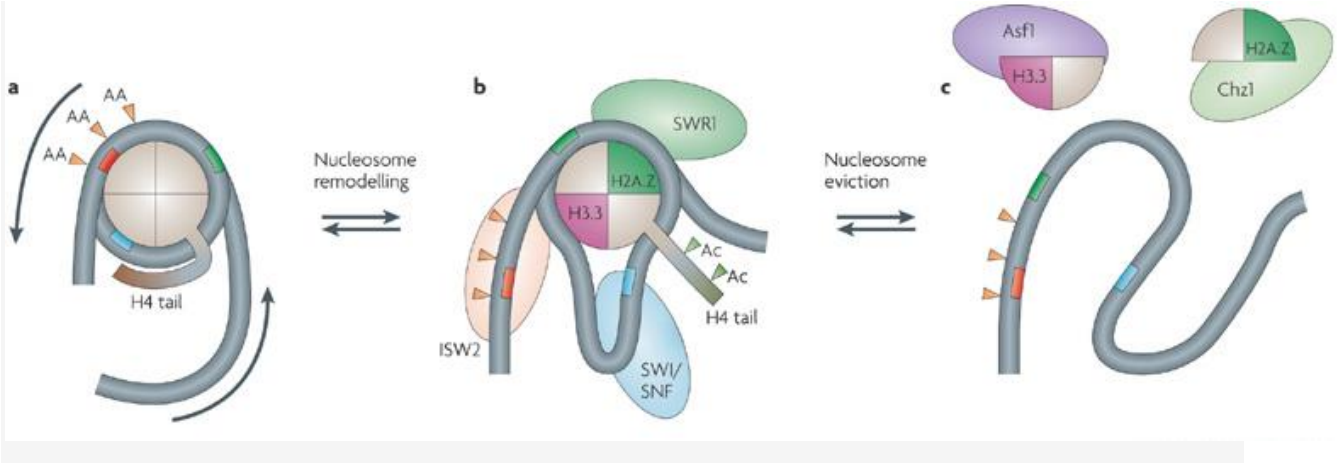


Figure and legend are from: Cizhong Jiang & B. Franklin Pugh , Nucleosome positioning and gene regulation: advances through genomics., *Nature Reviews Genetics* ,2009

Figure 2 Nucleosome state.

a | A stable nucleosome. **b** | A remodelled nucleosome. **c** | An evicted nucleosome. Three transcription factor binding sites are shown in red, green and blue, respectively. The red and blue sites become accessible only during

remodelling, either by nucleosome sliding, as indicated by the arrows in **a**, or by chromatin remodelling complexes (for example, ISW2, SWR1 and SWI/SNF) that 'extract' DNA from the nucleosome surface, as shown in **b**. Owing to rotational phasing, the green site is always accessible in the various states. Nucleosome eviction (**c**) might be necessary to assemble a pre-initiation complex and to transcribe the underlying DNA. Anti-silencing function 1 (Asf1) and H2A.Z-specific chaperone (Chz1) are examples of histone chaperones. Ac, acetylation

1.1.2 Effect of locus-dependent DNA methylation: CpG island

The DNA methylation is the covalent addition of a methyl group onto carbon 5 of the cytosine residue (5mC) of a CpG dinucleotide, catalyzed by DNA methyltransferas (DNMTs) enzymes [15]. More than half of all genes have regions with high concentration of these CpG dinucleotides, called “CpG islands” (CGIs) [16]. These CGIs are at least 200 base pair long, and are localized in the 5' promoter region of 50% genes. In a normal differentiated cell, there is a global hypermethylation of CpG islands, whereas promoter CG Islands of active genes are hypomethylated [16]. Genome-wide studies of the methylome with modern techniques like bisulfite-sequencing, have demonstrated that the position of the methylation influences genes control in different ways. For example, methylation near TSS blocks gene expression, but methylation in the gene body stimulates transcription elongation, and it may regulate splicing. Other important regions are the so called “CpG island shores”, located up to 2 kb upstream of some promoter CGIs. As for the TSS, also the methylation of these regions correlates with gene expression (Figure 3). DNA methylation represses gene expression through direct interference with the binding of specific transcription factors and with the binding of complexes that can positively remodel the chromatin and through the direct binding of methyl-binding domain proteins (MBDs) [15]. These MBDs, act together with the other repressor complexes such as histone methyltransferases and histone deacetylases, in order to obtain chromatin reconfiguration and gene silencing [17]. CGIs DNA methylation plays a fundamental role in the repression of a set of genes germ cell-specific, genomic imprinting [18] X chromosome

inactivation [19] and regulation of tissue aging [20]. The methylation in regions with repetitive elements such as centromeres and transposable elements, like LINE, regulates chromosomal stability [21] and genome stability, too. DNA methylation is finely regulated by mechanisms for add , maintain and remove methyl group. The methyltransferases enzymes DNMT3A and DNMT3B promote de novo DNA methylation in early development [22]. DNMT1 instead is the "methyltransferase maintainer", given that it alone maintains the DNA methylation pattern [19]. DNMT1 preferentially methylates only 1 of the 2 DNA strands and is used to maintain firmly the degree of DNA methylation through cell division [22]. All the three DNMTs are necessary for both embryonic and neonatal development. Methylation activity is essential for viability of somatic [23] or cancer cells [24].

DNA methylation is a plastic event and can be actively or passively regulated also by demethylating agents, such as activation-induced cytidine deaminase (AID), thymine DNA glycosylase (TDG) and the ten-eleven translocations (TET) methylcytosine.

An altered activity of these enzymes can lead to alteration of gene's activation [25,26,27] or genomic instability events underlying the human carcinogenesis [28].

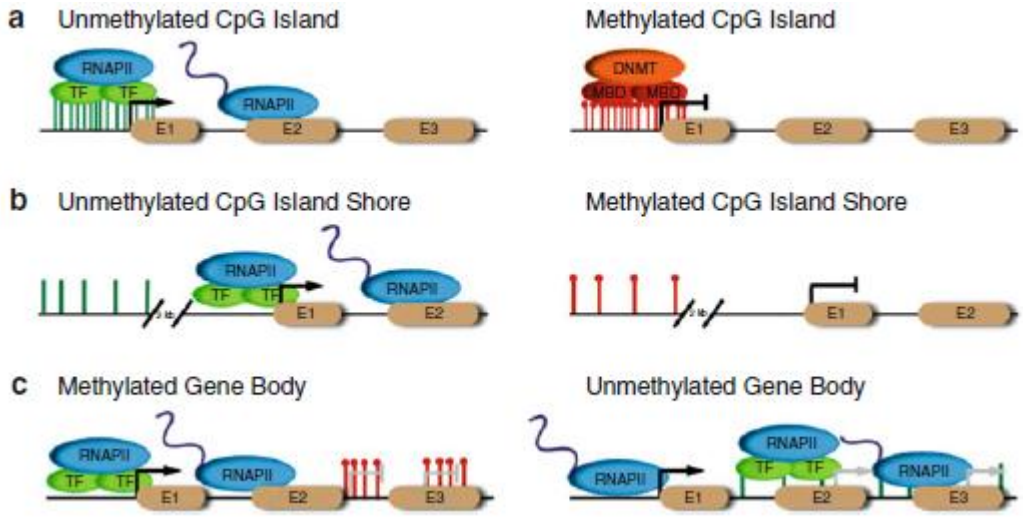


Figure and legend are From: Miller JL, Grant PA., The role of DNA methylation and histone modifications in transcriptional regulation in humans, Subcell Biochem. 2013

Figure3: Effects of DNA methylation throughout the genome.

DNA methylation is found at inter- and intragenic regions throughout the genome. DNA methylation dependent transcriptional activity is contingent on CpG dinucleotide genic location and density. Normal methylation events and subsequent effects are shown on the left. (**a**) CpG islands at promoters are normally unmethylated resulting in gene expression. However, aberrant hypermethylation at the same promoter results in corepressor complex recruitment and subsequent gene repression. (**b**) Intragenic regions characterized by scattered CpG dinucleotides located 2 kb upstream of the promoter called CpG island shores are regulated in the same manner as (**a**). (**c**) DNA methylation within the gene body prevents initiation of transcription from spurious sites in the gene. If unmethylated, these sites become transcriptional start sites resulting in an incorrect product

1.1.3 Histone Modifications

Histones are small proteins, with a molecular weight of 11_20 kDa, constituted by a globular domain and charged tails which have numerous lysine and arginine residues. Histones regulate the chromatin dynamics either by altering the structure, through changing the electrostatic charge and influencing nucleosome positioning, or providing sites for the recognition of proteins involved in chromatin remodelling through specific modifications. [29,30]. Histone modifications consist in numerous covalent reactions involving the histone N-terminal tails, and form the code (known as histone code) that regulates DNA wrapping around the histone proteins themselves. Histone tail modifications include methylation, acetylation, ubiquitination, sumoylation, phosphorylation and ADP ribosylation [31] of specific amino acids. The most studied modifications are methylation and acetylation of specific lysine residues on histones H3 and H4. In these N-terminal tails, the adding of an acetyl group compensates the positive charge of the lysine residues, reducing the tight bond between DNA and histones and opening the chromatin configuration enough to be accessible and leading to gene transcription [32]. These histone modifications are regulated by the cross-talk between histone acetyltransferases (HATs) and deacetylases (HDACs), which, respectively, introduce and remove an acetyl group.

According to cellular location and function, HATs can be divided into 2 distinct groups: the nuclear A-type HATs and the cytoplasmic B-type HATs [33]. The firsts affect gene transcription, whereas the cytoplasmic HATs type acetylates nonhistone proteins. Based on the similarities of the sequence, HATs can be classified into three categories: the MYST families, p300/CBP and Gcn5/ PCAF. These families generate a network of interplay and through the acetylation of target promoter regions and general acetylation, have an important role in differentiation, development and cell cycle progression [34]. In addition to these chromatin modifying enzymes, there are chromatin binding proteins, called epigenetic ‘‘readers’’, like the bromodomain proteins which can read lysine acetylation marks. They are involved in the regulation of several cellular processes including signal transduction, cell cycle regulation, apoptosis, and cell growth [35]. HDACs can be either cytoplasmic or nuclear and can deacetylate both non nonhistone and histone proteins. Cytoplasmic deacetylation leads to post-translational modifications of chaperone proteins and transcription factors. Furthermore, it can affect several important pathways, such as nuclear factor kappa B (NF-kB) and the gene of phosphatase and tensin homolog (PTEN)- dependent pathways [36,37]. The catalytic activity of HDACs involves the association with other complexes, such as the sirtuins, very important for the maintenance of the stemness [38].

Histone methylation is a dynamic modification, regulated by two classes of enzymes: histone methyltransferases (HMTs) and histone demethylases (HDMTs), which, introduce and remove methyl groups, respectively. These enzymes can promote or repress genes, depending on the specific substrate (figure 4). The arginine and lysine methylation on both histones or nonhistone regulatory regions or proteins such as TFs, modulates chromatin structure and also gene expression [39]. For example, methylation of histone H3 lysines 4 and 36 is associated with active genes expression, (with transcription activity and elongation, respectively), whereas methylation of histone H3 lysines 9 and 27 correlates with gene silencing.

Histone methyltransferases and demethylases are often part of a larger protein complexes regulating gene transcription. For example, the H3K27me3 is associated to the activity of the chromatin repressor complex Polycomb. Polycomb group (PcG) controls the accessibility of regulatory elements on the transcription machinery, through repression of proteins [29].

Finally, histones can be targeted by posttranslational modifications such as ubiquitination, ADP-ribosylation and phosphorylation, which also play an important role in gene regulation. For example, the phosphorylation of serine 10 is inversely correlated with lysine methylation [40]. Histone modifications works together with chromatin remodelers and DNA methylation creating an "epigenetic code" which characterized and defines genomic regions based on their activity status. Regions transcriptionally inactive have histones methylated and deacetylated on Lys 9 and 27 and high levels of methylated DNA, whereas genomic regions transcriptionally active are hypomethylated at level of DNA and have a chromatin structure globally acetylated and specifically methylated on Lys 4 and Lys 36. The alteration of histone modifiers activity caused by mutations may affect the whole epigenetic regulation mechanisms resulting in aberrant gene expression patterns.

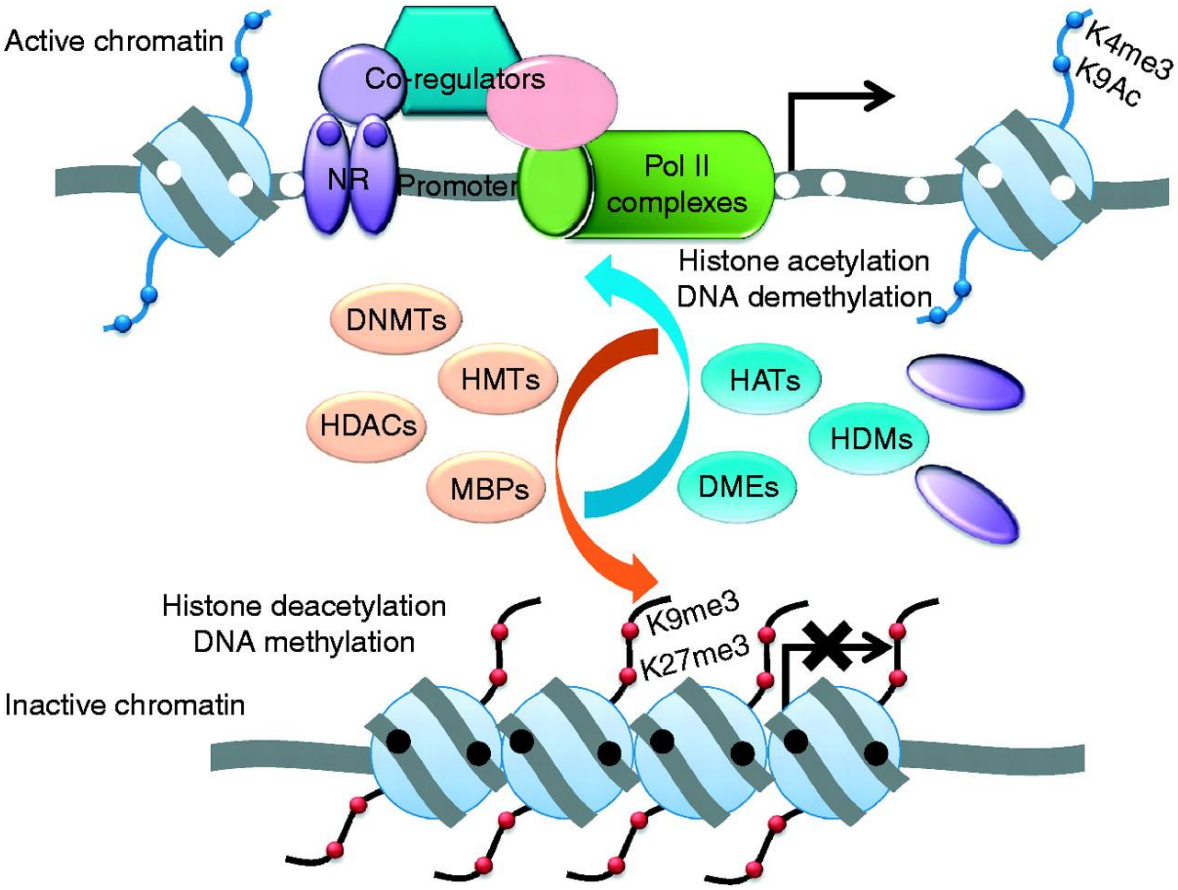


Figure and legend are from: Xiang Zhang and Shuk-Mei Ho, Epigenetics meets endocrinology, J Mol Endocrinol February 1, 2011

Figure 4: Histone modifications regulating gene activation.

DNA methylation and histone modification are two major epigenetic mechanisms that corroborate in regulating endocrine-related gene expression. Packaging genes into active or inactive chromatin determines whether they are transcriptionally accessible or not. The N-termini of histones have specific amino acids that are sensitive to posttranslational modifications, which contribute to chromatin status. Moreover, hypermethylation of promoter is associated with transcriptional silencing due, in part, to the loss of affinity for transcriptional factors such as the nuclear receptor (NR) and accessibility by the transcriptional machinery as represented by RNA Pol II complexes. The inactive chromatin has increased affinity for methylated DNA-binding proteins (MBPs), which further recruit histone deacetylases (HDACs), DNA methyltransferases (DNMTs), and histone methyltransferases (HMTs), and other corepressors. Methylated promoters are associated with unique repressive histone markers, which classically include trimethylation of histone 3 (H3), lysine (K) 9, and H3-K27. In contrast, unmethylated promoters are associated with gene transcription. They have increased affinity for histone acetylases (HATs), histone demethylases (HDMs), DNA demethylases (DMEs; e.g. DNA *N*-glycosylase), and histone marks associated with

active chromatin, including acetylated H3-K9 and trimethylated H3-K4. Nucleosome remodeling such as repositioning and ejection in promoter results in gene transcription (bent arrow). Me, histone methylation; Ac, histone acetylation; black filled circle, methylated CpG dinucleotides; white filled circle, unmethylated CpG dinucleotides; filled purple circle, hormone or endocrine disruptors that bind to NR.

1.1.4 microRNAs

miRNAs are short (about 19-25 nucleotides (nt) in length) non-coding RNAs (ncRNAs) that by binding to specific mRNA targets, modulate their expression, through degradation and/or translational inhibition [41] . Thus, microRNAs are the only epigenetic mechanisms that regulate gene expression at the post-transcriptional level. miRNA genes are often located in fragile regions and deleted sites in human genome, in introns and rarely in exons and can be organized in monocistronic or polycistronic units [42,43]. One miRNA alone may regulate hundreds mRNAs, thus modulating gene expression networks and also whole pathways. Indeed miRNAs are involved in many contexts such as development and maintenance of tissue differentiation [44], inflammatory response [45, 46], autoimmune diseases (47) and also in cancer [48].
miRNA genes are transcribed by RNA polymerase II and processed into mature miRNAs through the canonical miRNA biogenesis pathways (figure 5). The RNA polymerase II generates a long hairpin double strand RNA (pri-miRNA) that is cleaved by endonuclease Drosha, a member of RNAse III family, generating a precursor miRNA (pre-miRNA) 70 nt-long. There is also a non canonical biogenesis, in which the pre-miRNA is generated through mRNA splicing machinery, avoiding the endonuclease digestion. The pre-miRNAs is exported in the cytoplasm by the nuclear export protein exportin 5 and here is further processed by another RNase III enzyme, Dicer, to a double strand miRNA 22 nt-long. This mature miRNA is loaded into the RNA-induced silencing complex (RISC), a functional ribonucleoprotein complex which mediates the post-transcriptional gene silencing of the miRNAs (figure 5).

RISC is composed by multiple protein factors, like Argonaute proteins, which are the key catalytic enzymes of the complex. Argonaute proteins, like Ago II, bind miRNAs and are essential for the regulation of their target mRNAs. The binding of the miR-RISC complex to 3'UTR of target mRNA is regulated by the "seed region", a sequence of 2–8 nucleotides, located at the 5′ end of the mature miRNA. miRNAs may silence gene through mRNA destabilization and decay, accelerating uncapping and deadenylation and/or translational repression, inhibiting translation initiation, or translation post-initiation [49-52]. In the deadenylation process the polyA tail is removed from the mRNA, through the activity of deadenylases, which induces mRNA instability and cleavage. The inhibition of the initiation factor 4E (EIF4E) leads to block of mRNA translation. In mammalian, mRNA destabilization is the dominant way of inhibition operated by miRNAs. This occurs possibly with the involvement of P body proteins, which are cytoplasmic processing bodies enriched with proteins and enzymes that sequester the messenger from the translational machinery and degrade it.

Recent studies demonstrated that several miRNAs, more than 100, are produced by cells of the immune system and that they can modulate, through the molecular pathways, the function and development of innate and adaptive immune responses.

Thus, in the last years some pharmaceutical companies have tried to use miRNAs for therapeutic treatment.

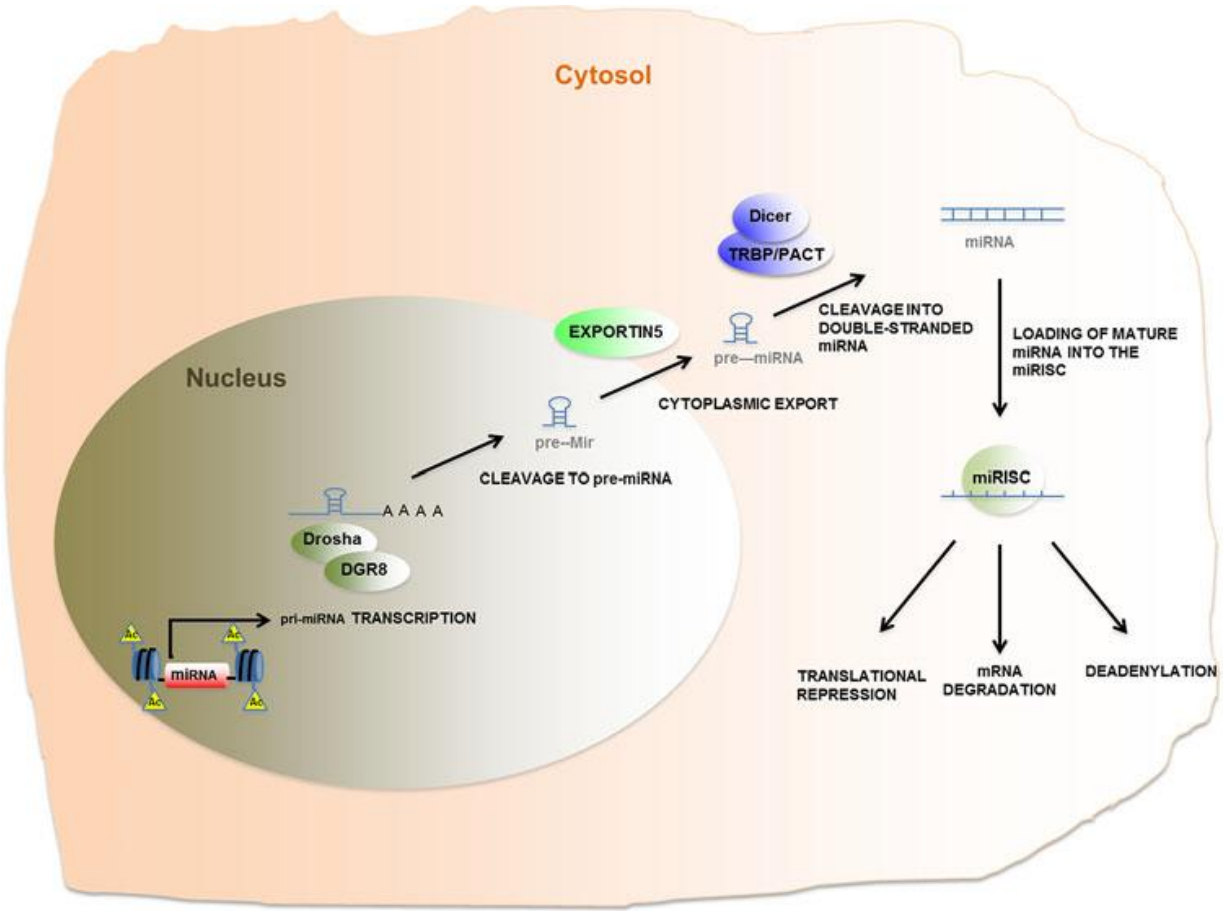


Figure and legend are from Roberto Papait,Carolina Greco, Paolo Kunderfranco, Michael V. G. Latronico, Gianluigi Condorelli, Epigenetics: a new mechanism of regulation of heart failure? Basic Res Cardiol 2013.

Figure 5: Biogenesis of microRNAs

Transcription of primary micro RNA (Pri-miRNA) from miRNA genes is followed by cleavage to precursor mRNA (Pre-miRNA) by the Drosha nuclear RNase III. The Pre-miRNA is then exported to the cytoplasm by exportin via nuclear pore. In the cytoplasm, Pre-miRNA is further processed by RNase activity of Dicer to the mature micro RNA duplex. The duplex loads onto Argonaut ribonucleases in the RISC complex and separates. One of the mature miRNA strands (red strand) mediates small interfering RNA silencing by degrading the target mRNA or interfering with translation. The outcome of RISC formation varies with the degree of complementarity of the seed sequence of miRNA and 3' untranslated regions (UTR) of the target mRNA.

1.2 Enhancers and gene regulation

Enhancers are short DNA cis-regulatory elements that enhance the transcription of target gene promoters influencing the destiny of cells during development and differentiation [53]**.** Enhancer sequences are characterized by short different DNA motifs containing binding sites for specific transcription factors. These motifs confer to enhancers their specificity of action: an enhancer binds different co-activators and co-repressors molecules, in a manner tissue and stimuli dependent, and their combined effect determine the intensity of enhancer effect. A functional characteristic of enhancers is that they act independently by the orientation and distance to their target genes: they may remodel chromatin to form a loop on the gene target, also located at distances of several megabases [54].

The physical interaction between specific enhancers and target promoters can be investigated through the chromosome conformation capture (3C) technique [55, 56]. This loop is the result of interplay between negatively regulatory elements (insulators) and activators, which interact with the RNA polymerase II (Pol II) and with transcriptional coactivator complexes, like p300, to bind enhancer loops together with the transcription apparatus [57]. Indeed, enhancers modulate components of the basal transcription machinery [58, 59, 60] and can regulate Pol II activity at transcript initiation, elongation, or termination. The first step of transcription is the bind of RNA Pol II and cofactors to gene promoters, forming the Transcription preinitiation complex (PIC), after which there is the elongation phase and the Polymerase II complex moves across gene body, transcribing the gene (figure 6).

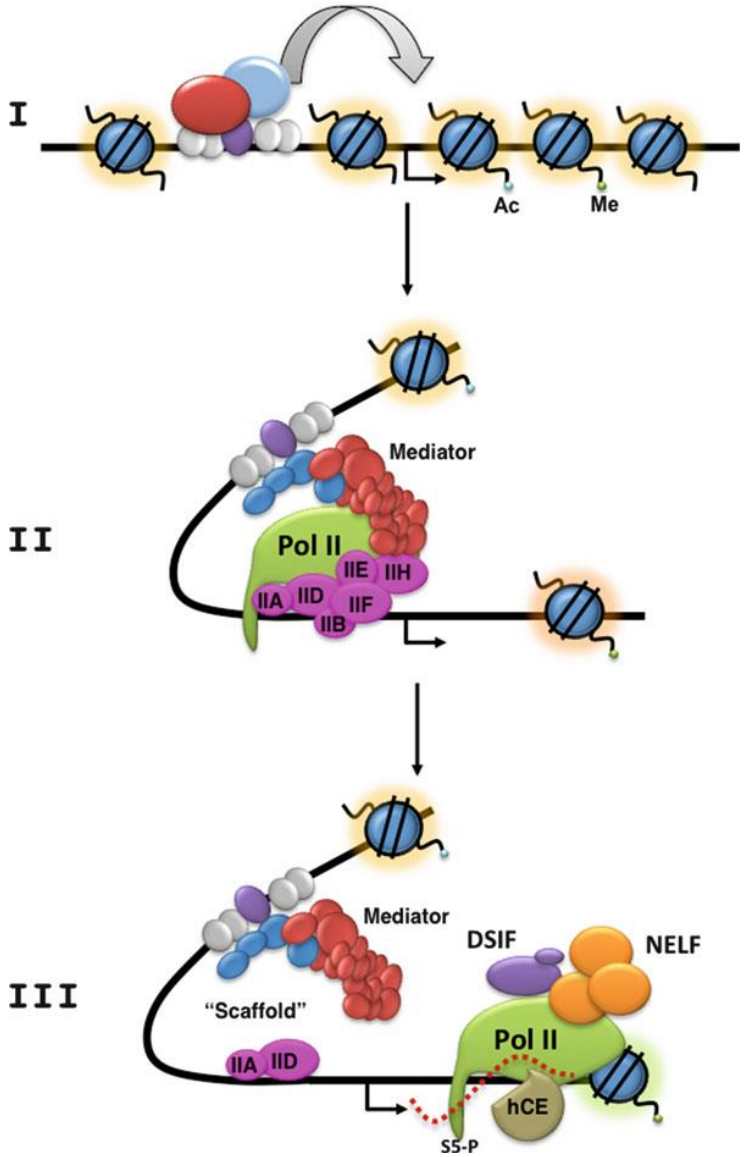


Figure and legend are from Epigenetics: Barrero MJ, Malik S. The RNA Polymerase II Transcriptional Machinery and Its Epigenetic Context, book Epigenetics: *Development and Disease 2012*

Figure 6 :Transcription by Pol II is a multi-step process . The figure depicts a simplified transcription activation pathway that commences when transcriptional activators bind to their cognate sites in the regulatory region of the gene that may be buried in chromatin. These factors recruit a series of chromatin coactivators that can both covalently modify nucleosomes at specific histone residues and mobilize the nucleosomes via ATP-requiring reactions. The resulting intermediate (I) contains chromatin that is characterized by distinct covalent modifications such as acetylation (Ac) and methylation (Me); some nucleosomes may be "evicted". The activators then recruit Mediator (intermediate II). Although the intact Mediator consisting of the core and the kinase module might be recruited with subsequent loss of the kinase module as the PIC matures, only the core Mediator is shown. PIC assembly entails entry of the various GTFs and Pol II. After transcription initiation, Pol II clears the promoter. Prior to fully entering the elongation phase , Pol II, which by now is phosphorylated at Ser5, may pass through a capping

checkpoint. The scaffold complex (intermediate III) containing a subset of GTFs and Mediator remains behind at the promoter and can contribute to subsequent rounds of transcription. At the capping checkpoint, Pol II becomes associated with elongation factors including DSIF and NELF. The capping enzyme (hCE) modifies (7MeG) the nascent RNA. Pol II is released from this pause through recruitment of P-TEFb.

This elongation can be inhibited by blocking Pol II. Enhancers promote transcription both by favouring PIC formation on the promoter and also by releasing paused Pol II, activating specific complexes and allowing elongation [61]. Moreover, recent studies showed that RNA Pol II binds not only TSS of promoter regions but also enhancers, resulting in the generation of a transcript, called eRNA [62]. This eRNA can modify chromatin structure on target promoter, increasing target gene expression. An active enhancer is nucleosomes free, thus the DNA is accessible to transcription factors. Furthermore, the different state of enhancer activation can be detected by specific histone modification marks located on the nucleosomes in proximity of the enhancer (figure 7). An active enhancer is characterized by the present of histone H3 lysine 4 monomethylation (H3K4me1) and the open chromatin mark H3K27 acetylation (H3K27ac). An intermediate phase of activation is characterized by the presence of a “bivalent domain”: the nucleosomes show both H3K4me1 and one of the closed chromatin mark histone H3 lysine 9/27 threemethylation (H3K9me3 or H3K27me3). This kind of enhancer is called "poised" and its activity is reduced by the effects of chromatin repressor complexes like Polycomb and is recognised by the presence of H3K27me3 and H3K9me3. These genes are usually found in stem cells, whereas during differentiation, they can become fully activated (loosing H3K9/27me3) or repressed (loosing H3K4me1) [63–65].

In contrast, an inactive enhancer localizes with nucleosomes and thus, does not have transcription factors.

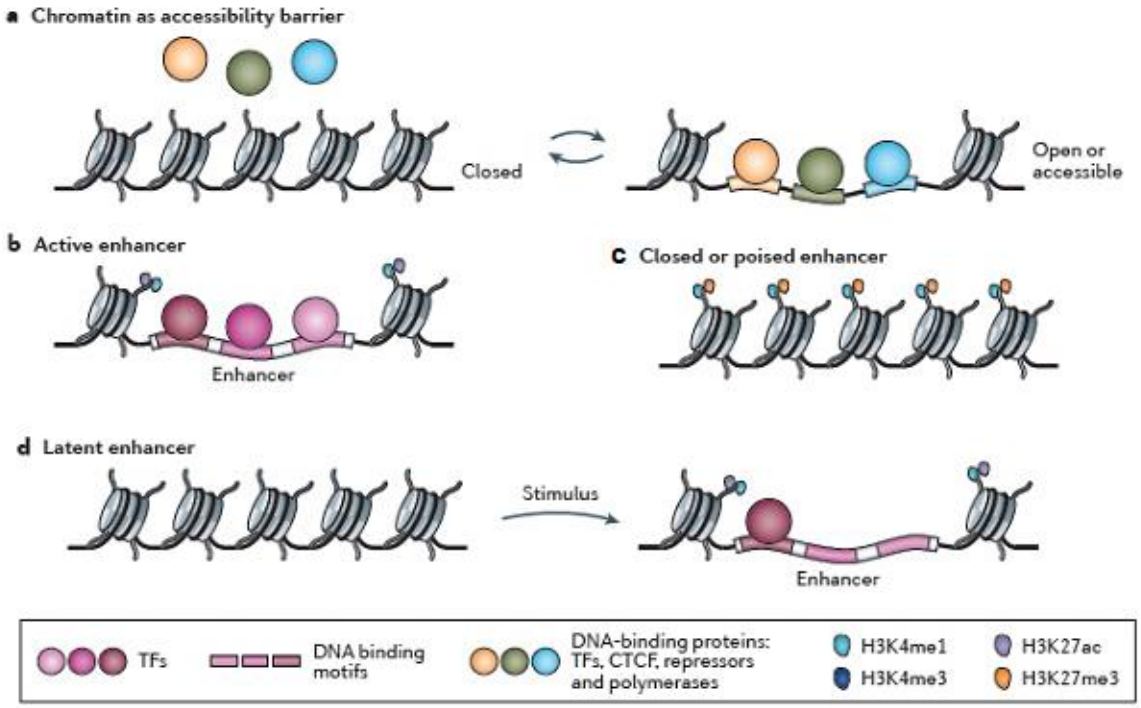


Figure and legend are from: Daria Shlyueva, Gerald Stampfel and Alexander Stark, Transcriptional enhancers: from properties to genome-wide predictions, Nature 2014.

Figure 7: Chromatin accessibility and histone marks at regulatory elements.

a) Chromatin is shown as a 'gatekeeper' for transcription factor (TF) binding and enhancer activity. Densely positioned nucleosomes can restrict access for transcription factors (both activators and repressors), RNA polymerase II (Pol II) and other proteins. Accessible (that is, nucleosome-free) regions can be bound by these proteins, which define and mediate the identity of a region. The transition from 'open' to 'closed' chromatin, and vice versa, is determined by regulatory proteins, including pioneer transcription factors. Insulator proteins and other architectural proteins also bind to open regions, and they make up a substantial proportion of sites that are accessible across multiple cell types. **b)** Histones that flank active enhancers are often marked by histone H3 acetylated at lysine 27 (H3K27ac) and H3 monomethylated at lysine 4 (H3K4me1. **c)** Some closed or poised enhancer regions can bear the active H3K4me1 and the repressive Polycomb protein-associated H3K27me3 marks. **d**) Latent enhancers are located in closed chromatin and are not pre-marked by known histone modifications. However, in the presence of external stimuli the DNA becomes accessible, and flanking nucleosomes acquire H3K4me1 and H3K27ac marks.

1.3 Epigenetics in inflammation-associated diseases

In the last ten years several studies showed the involvement of aberrant epigenetic modifications in the aetiology of inflammatory and autoimmune diseases. There is a strong interplay between epigenetic and inflammation. Different studies have demonstrated that inflammation can modify epigenetic machineries, enhancing the whole genome DNA methylation [66]. The inflammation induces the epigenetic reprogramming that leads to aberrant immune responses. For example, the activity of the NF-kB transcription factor, important for the regulation of immune responses, including the differentiation of B and T cells, haematopoiesis and acute inflammatory responses, is associated to alterations of the activity of epigenetic regulators, like DNA Methyltransferases and Histone Methyltransferases and Polycomb group [67-70]. Moreover, epigenetic mechanisms themselves, can modulate the inflammatory response and thus, the inflammation-induced transcription. In general, the epigenetic control of transcription factors and key cytokines regulating the cells of the innate and adaptive immune systems is important in the differentiation, development, function and phenotype of DCs, neutrophils, monocyte/macrophages, and Th cells. For example, in Th cell differentiations, epigenetic mechanisms like histone modifications associated with silent heterochromatin and active euchromatin (H3K9me3 and H3K4me3 respectively), affect the production and release of IFN-γ and IL-4. Aberrations of these epigenetic modifications are associated with various diseases, such as rheumatoid arthritis, multiple sclerosis, type 1 diabetes, inflammatory bowel disease, systemic lupus erythematosus, and asthma.

The epigenetic machinery is a plastic mechanism that changes in a specific manner in response to environmental factors. These epigenetic variations can modify or influence different pathways, including the immunity responses and also lead to alterations of gene regulation mechanisms. Thus explain the complexity of human diseases (Figure 8).

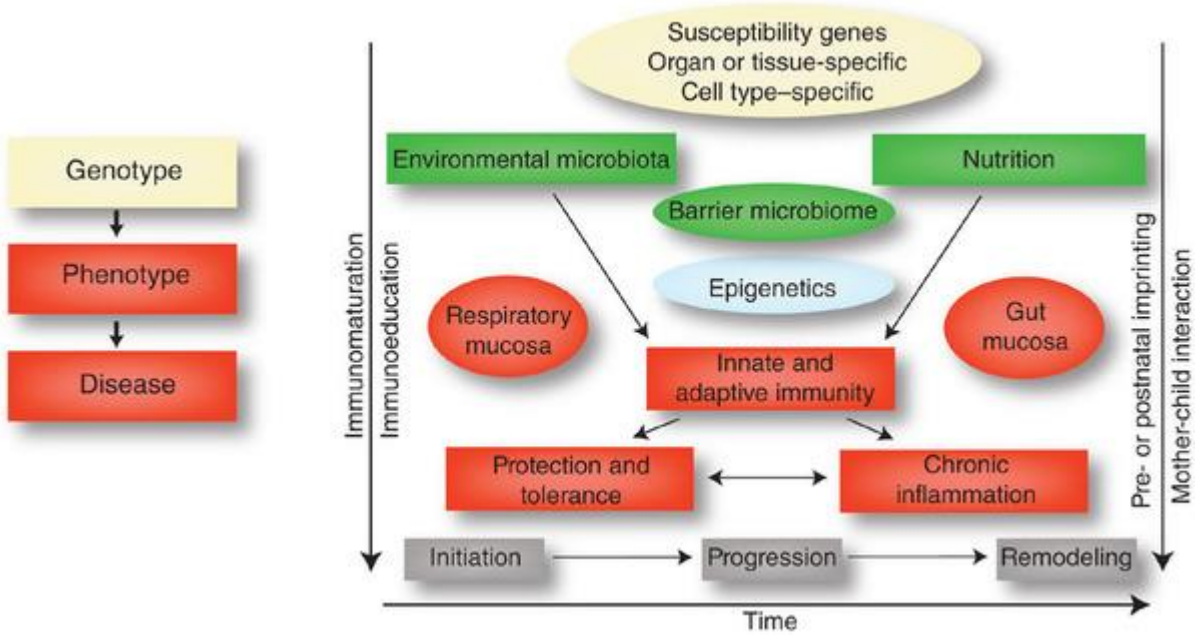


Figure and legend are from: Harakd Renz et al., Gene-environment interactions in chronic inflammatory disease, Nature Immunology 2011

Figure 8: A new paradigm for the development of chronic inflammation.

The traditional simplistic paradigm of chronic inflammatory disease development has been revised to a more complex understanding of the cornerstones of these conditions. Epigenetics (blue) represents a key mechanism linking genetic components (yellow) and the environment. Disease development depends on intimate interaction between intrinsic mechanisms (red) and extrinsic mechanisms (green) in a temporal and spatial way.

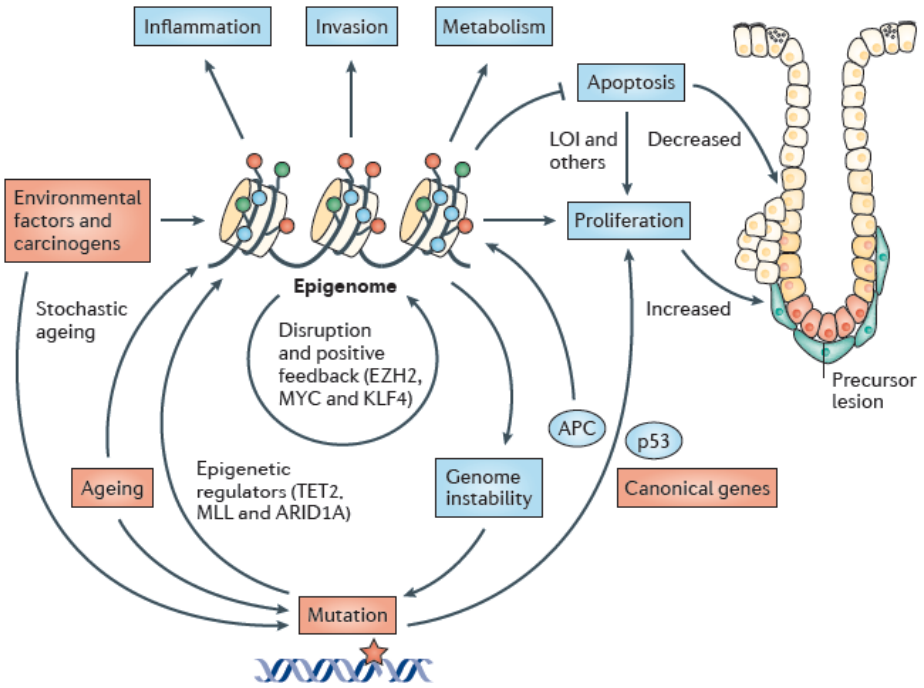
1.4 The Epigenomics of Cancer

Epigenetic dysregulation correlates with all the processes generating cancer. Indeed, together with mutations, also stochastic epigenetic variations increase the susceptibility to cancer. Alteration of epigenetic mechanisms can be caused by mutations in the genes that encode for these mechanisms or by epigenetic modifications in such genes, generating a positive feedback. Indeed epigenetic changes can occasionally result from single nucleotide polymorphisms in proximity to promoter regions. Feinberg's group hypothesized that the dysregulated epigenetic mechanisms work together with mutations of the crucial genes which alter pathways that

leading to cancer development [71] (Figure 9). Moreover, the epigenetic changes themselves contribute to increase mutation frequency. The mutations in genes controlling epigenetic were found in rare solid tumours, and in haematopoietic malignancies. Cancer cells show some global epigenetic aberrations, like DNA hypomethylation, histone deacetylation and hypomethylation, down-regulation of miRNAs. More in details, focusing on histone modifications in cancer, a general loss of histone H3 lysine 4 trimethylation (H3K4Me3) and lysine 9 acetylation (H3K9Ac) was shown. Furthermore, the gain of histone H3 lysine 27 trimethylation (H3K27me3) and lysine 9 trimethylation (H3K9me3) at specific tumor soppressor gene promoters silenced them, contributing to tumorigenesis [72]. Indeed, overexpression of Polycomb group promotes tumor growth both in vitro and in vivo [73], and is found in different cancers, such as lymphoma melanoma, breast and prostate cancers [74]. Moreover, histone H3 lysine 9 methyltransferase is important for carcinogenic initiation and progression [75].

In cancer there is a global alteration in miRNA expression [76]: most miRNAs are down-regulated by epigenetic or genetic events, whereas some, depending on the kind of tumor, are upregulated. For example, miR-17-92 cluster is overexpressed in chronic myeloid leukemia and in breast and lung cancers, favouring tumour development, silencing the transcription factor E2F1, which is a major cell cycle regulator. Aberrant DNA methylation is the most studied epigenetic modification in cancer. For example, genes bound to Polycomb Repressive Complex proteins in embryonal cells, which DNA is methylated in cancer, are of great interest.

All these alterations provide a growth advantage to neoplastic cells respect to normal cells, leading to uninhibited cellular proliferation, apoptotic deficiency and tumorigenicity (Figure 9).



Figured and legend are from: Winston Timp, Andrew P. Feinberg, Cancer as a dysregulated epigenome allowing cellular growth advantage at the expense of the host, Nature Reviews Cancer 2013

Figure 9: Collaboration of epigenetic modification and mutation in the hallmarks of cancer.

The epigenome sits at the intersection of the environment, genetic mutation and tumour cell growth. Environmental factors, such as carcinogens or diet, as well as injury and inflammation, cause epigenetic reprogramming. The epigenome also accumulates damage stochastically and through ageing. The machinery for maintaining epigenetic integrity can be stably disrupted in either of two ways: by mutation or by epigenetic change itself with positive feedback.

1.4.1 **DNA methylation in Cancer**

Several studies show the key roles of DNA methylation in tumors. Aberrant DNA methylation is transmitted among generations comporting a growing increase of epigenetic alterations in the daughter cells. Genome-wide DNA methylation studies in colon cancer, versus healthy colon samples showed a demethylation in some regions, megabases long, located across multiple chromosomes [77-79]. Some of these demethylated domains are associated with proteins in the nuclear lamina. DNA demethylation can occur in an active or passive manner [80, 81]. Active

DNA demethylation is operated by enzymes that modify methylation through oxidation, hydroxylation, or deamination and that are independent from DNA replication [82]. Important regulators of demethylation are activation-induced cytidine deaminase (AID) and ten-eleven-translocation (TET), which induces hydroxylation [83]. In contrast, passive DNA demethylation occurs during DNA replication, when the mechanisms responsible to maintain DNA methylation are impaired.

Furthermore, genome of cancer cell is characterized by specific hypermethylated regions corresponding to CpG islands of promoter oncosuppressor genes [28, 84, 85, 86]. For example, in various cancers DNA hypermethylation occurs in genes controlling DNA repair, such as O6-methylguanine-DNA methyltransferase (MGMT) and the mismatch-repair gene MLH1, and cell cycle, like PTEN [87]. Hypermethylation of MGMT in cancer is associated to higher susceptibility to genetic mutations in critical genes such as KRAS and p53 [88]. Moreover, aberrant MLH1 promoter methylation induces microsatellite instability, which is a key factor in several cancers, like colorectal and endometrial cancers. The human colon cancer methylome shows similar hypo- and hypermethylation at conserved tissue-specific CpG island shores .

The alteration of DNA methylome is a consequence of alterations of the epigenetic machineries. Indeed, in different tumors, like colorectal cancer, DNMT1 mutations were found (Kanai et al., 2003), whereas DNMT3A alterations are common in acute myeloid leukemia and in myelodysplastic syndromes [89].

Also genetic mutations in Methyl-binding domain (MBD) proteins, such as MeCP2, have been reported in several cancers.

Aberrant DNA methylation is also a diagnostic biomarker in biopsy specimens and in body fluids such as sputum, serum, saliva, pleural or peritoneal effusions and urine. For example, p16 methylation is a possible biomarker to assess a patient’s risk to develop lung cancer. Thus the study of DNA methylation can be useful to prevent cancer, through the development of drugs

able to revert specific DNA methylation. Finally, DNA methylation is also used to assess response to chemotherapeutic agents and clinical outcomes [66].

2. Inflammation and cancer

2.1 Cancer Related inflammation: extrinsic and intrinsic pathways

Connections between cancer and inflammation were found since the nineteenth century, based on the observations that an inflammatory infiltrate was present in tumours, and that often tumors arise at sites of chronic inflammation. Epidemiological studies have shown that chronic inflammation has many ways to increase the risk of developing cancer [90] (in particular cervical, bladder, ovarian, prostate, intestinal, gastric, oesophageal and thyroid cancer). These ways include microbial infections (for instance infection with Helicobacter pylori, that is associated with gastric mucosal lymphoma and gastric cancer), autoimmune diseases (such as inflammatory bowel disease that is associated with colon cancer) and various inflammatory conditions. Furthermore, the overexpression of inflammatory cytokines or the transfer of inflammatory cells promotes the development of tumours in preclinical models.

The risk of developing certain cancers, like colon cancer, can be reduced by non-steroidal anti-inflammatory drugs [91-93], that reduce also the mortality associated to these cancers. On the same line, the incidence and growth of cancer can decrease through the targeting of inflammatory cells, the principal transcription factors involved in inflammation (such as nuclear factor-κB , NF-κB and STAT3) and inflammatory mediators (cytokines and chemokines, such as interleukin 1 beta , IL-1β and tumour necrosis factor-α, TNF-α).

The hallmarks of Cancer-related inflammation (CRI) are the presence of inflammatory mediators (like prostaglandins, chemokines and cytokines) and inflammatory cells in tumour

tissues, angiogenesis and tissue remodelling, like those observed in chronic inflammatory responses and tissue repair.

Recent studies have demonstrated that also complement effectors, like C5a, are potent proinflammatory mediators involved in CRI and that they can enhance tumors growth [94]. The proteins of the complement cascade are abundant in biological fluids of patients with different types of tumor [95–97,] and elevated complement levels correlated with lung tumor size [98].

CRI can be conceptually divided in intrinsic and extrinsic inflammation.

The intrinsic pathway is regulated by genetic events, like oncogene mutation or overexpression, which cause cancer and orchestrate the construction of an inflammatory microenvironment. The extrinsic pathway, activated by infectious or inflammatory conditions, can increase cancer risk . (figure 10). Classical examples of cancers associated to the extrinsic pathway are mucosa-associated lymphoid tissue (MALT) lymphoma and gastric cancer, triggered by persistent Helicobacter pylori infection or colorectal cancer associated to IBD, or hepatocellular carcinoma (HCC) associated 2 infections with hepatitis C (HCV) or B (HBV) viruses.

By investigating why inflammatory mediators and cells are present in the microenvironment of all tumours independently from epidemiological basis for inflammation, the intrinsic pathway was discovered. Various studies demonstrated that an early genetic event that is sufficient and necessary for the starting of human tumour development, directly promotes the formation of an inflammatory microenvironment [99]. An important mechanism that promotes intrinsic inflammation is the response to cellular stresses, in particular DNA damage.

The most frequently mutated oncogenes in human cancer are the members of the RAS family which activate the oncogenic components of the RAS–RAF signalling pathway that, in turn, induce the production of inflammatory chemokines and cytokines that promote inflammation [100–102].

Another relevant oncogene is MYC, which encodes for a transcription factor over expressed in many human tumours. The deregulated expression of MYC promotes and maintains any key

aspects of the tumour phenotype. MYC promotes the cell-autonomous proliferation and also induces a reconfiguration of the extracellular microenvironment, with the help of inflammatory cells and mediators. The transcriptional program activated by MYC stimulates also the production and release of several chemokines involved in the recruitment of mast cells. Mast cells drive angiogenesis, and in response to IL-1β they sustain tumour growth and new blood-vessel formation [103]. The studies of MYC and RAS family members demonstrated that dominant oncogenes trigger the formation of a microenviroment that promotes the tumour (the intrinsic pathway).

Studies in mouse models of pancreatic tumor have demonstrated that to induce invasive ductal carcinoma and pancreatic intra-epithelial neoplasia both mutated Kras and mild chronic pancreatitis (respectively the intrinsic and the extrinsic pathway of CRI) are required.

In conclusion, several types of gene, such as tumour suppressor proteins, RAS and RAF and transcription factors, coordinate different inflammatory transcriptional programs that have in common the recruitment of cells of myelomonocytic origin and a link to angiogenesis.

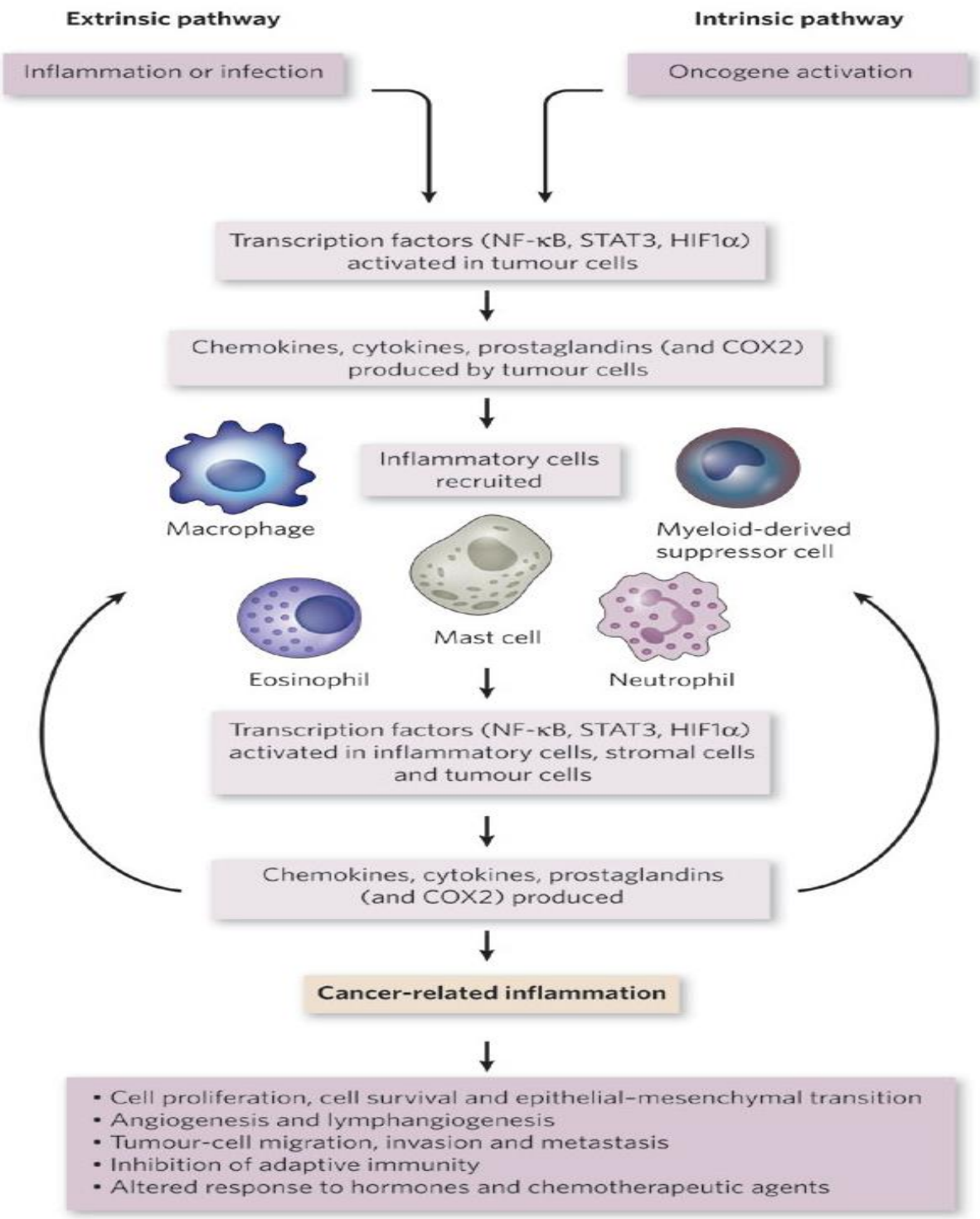


Figure and legend are from: Alberto Mantovani, Paola Allavena, Antonio Sica & Frances Balkwill, Cancer-related inflammation, Nature 2008

Figure 10: Pathways that connect inflammation and cancer.

Cancer and inflammation are connected by two pathways: the intrinsic pathway and the extrinsic pathway. The intrinsic pathway is activated by genetic events that cause neoplasia. These events include the activation of various types of oncogene by mutation, chromosomal rearrangement or amplification, and the inactivation of tumour-

suppressor genes. Cells that are transformed in this manner produce inflammatory mediators, thereby generating an inflammatory microenvironment in tumours for which there is no underlying inflammatory condition (for example, breast tumours). By contrast, in the extrinsic pathway, inflammatory or infectious conditions augment the risk of developing cancer at certain anatomical sites (for example, the colon, prostate and pancreas). The two pathways converge, resulting in the activation of transcription factors, mainly nuclear factor-κB (NF-κB), signal transducer and activator of transcription 3 (STAT3) and hypoxia-inducible factor 1α (HIF1α), in tumour cells. These transcription factors coordinate the production of inflammatory mediators, including cytokines and chemokines, as well as the production of cyclooxygenase 2 (COX2) (which, in turn, results in the production of prostaglandins). These factors recruit and activate various leukocytes, most notably cells of the myelomonocytic lineage. The cytokines activate the same key transcription factors in inflammatory cells, stromal cells and tumour cells, resulting in more inflammatory mediators being produced and a cancer-related inflammatory microenvironment being generated. Smouldering cancer-related inflammation has many tumour-promoting effects.

2.2 Cancer Related inflammation: players and mechanisms

The extrinsic and intrinsic pathway of CRI converge inducing key regulatory transcription factors, like NF-kb and signal transducer activator of transcription-3 (STAT3). NF-κB operates downstream of the pathways mediated by the inflammatory cytokines IL-1β and TNF-α and of Toll-like receptor (TLR)–MyD88 signalling pathway. Furthermore, hypoxia conditions and genetic alterations of cells, like deletions, amplification or mutations, can also activate NF-kB in tumor cell. The NF-κB pathway is strongly controlled by inhibitors acting at various stages of the pathway. An example is TIR8 (SIGIRR), which can inhibit signalling acting on TLRs and the IL-1 receptor. In tumour cells NF-kB induces the expression of adhesion molecules, key enzymes in the pathway of prostaglandin synthetase (such COX-2), nitric oxide synthase (iNOS), inflammatory cytokines, and angiogenic factors. Moreover, in cells targeted by carcinogenic agents and in tumor cells, NF-kB promotes survival through induction of antiapoptotic agents (as BCL2). Different studies demonstrated the involvement of NF-kB in tumor initiation and progression in tissues like the gastrointestinal tract and the liver, in which

CRI typically occurs [104-106]. NF-kb and also other inflammatory transcription factors induce the release of inflammatory cytokines and chemokines by tumour cells. NF-kB has a role also in regulating tumour-infiltrating leukocytes. In particular, tumour-associated macrophages (TAMs), which are abundant in tumour microenvironments, have a defectively and delayed NF-κB activation [107]. This defectively activation of NF-κB in TAMs is caused by NF-κB p50 subunit, which negatively regulates the NF-κB pathway. P50 is also responsible for the protumour phenotype of these TAMs [108].

Like NF-κB, STAT3 regulates numerous oncogenic signalling pathways [109] and it is important for maintenance of NF-kB activation in tumors. STAT3 is constitutively activated both in immune cells and in tumour cells and is involved in inhibition of apoptosis and oncogenesis. STAT3 is the major controller of cell survival and proliferation, through regulation of the expression of Mcl-1, c-Myc, Bcl-2 and Cyclin D [110]. This transcription factor increases the capacity of tumours to evade the immune system, suppressing the immune response [111] and through inhibition of dendritic cells maturation. Finally, in the tumor microenvironment, the balance between IL-12 and IL-23 and so also the polarization of T-helper subsets is regulated by STAT3.

Tumour Necrosis Factor α (TNF α) improves tumor invasion and growth, angiogenesis, leukocyte recruitment and facilitates epithelial–mesenchymal transition. In addition, TNF-deficient mice are protected from skin carcinogenesis. Moreover, TNF family members mediate also immune suppression.

Together with TNF, also IL-1 affects some steps of the CRI cascade and can enhance metastasis. In a pancreatic islet tumor model, the inflammatory cytokine IL-1 promotes angiogenesis [112]. In transgenic mice, stomach expression of human IL-1β induces a spontaneous gastric inflammation and cancer, in correlation with early recruitment of myeloid-derived suppressor cells (MDSCs) [113]. IL-1β secreted by infiltrating leukocytes or malignant cells increases

tumor adhesiveness and invasion, angiogenesis and immune suppression, whereas IL-1ra negatively regulates these processes [114].

Chemokynes recruit and activate leukocytes, above all cells of the myelomonocytic lineage. For example, CXCL5 and CXCL12 attract myeloid-derived suppressor cells, which are strong suppressors of the response of adaptive immunity to tumours and facilitate directly metastasis. CC-chemokine ligand 20 (CCL20) attract dendritic cells and monocytes, CCL2, CCL5 and CCL18 recruit TAM in tumors [115- 116]. Furthermore, CCL9 recruit immature myeloid cells, for example in colon cancer models, acting through its receptor, CCR1.

A leukocyte infiltrate, varying in composition, size and distribution, is present in all tumours. Different studies have found that TAMs are among the most important infiltrating leukocyte and are involved in tumour progression. In addition to TAMs, related cell populations like myeloid dendritic cells, MDSCs and some types of monocytes, are involved in CRI [117]. At the beginning, macrophages involved in CRI are immune activated to have an antitumour activity, but during tumor progression they are educated to become protumoral [118]. During carcinogenesis, the microenvironment is dominated by growth factors and cytokines, released through stimulation of transcription factors mentioned above. The over production of these inflammatory factors provokes a switch from a tumour-inhibiting inflammatory response (Th1) to tumor promoting immune environment (Th2). TAMs are M0 and those with M2 phenotype suppress adaptive immunity, remodel tissues and promote both tumour growth and angiogenesis [119,120]. TAMs product macrophage-derived epidermal growth factor (EGF) or EGF family ligands and the tumor-cell-synthesized CSF-1, which induce directional invasion and migration of tumor cells. Thus, macrophages and tumor cells rapidly migrate along collagen fibres, reaching tumor cells clustering around blood vessels [121] . At the blood vessels, TAMs create an opening that permit the escape of tumor cells. Macrophages also produce other molecules that improve tumor cell invasion: osteonectin, which increases interaction between tumor cells and the extracellular matrix and thus migration; cathepsin proteases that modify the matrix and

thus release sequestered growth factors [122]; TGF-beta that promotes epithelial-mesenchymal transition of the invading tumor cells. TAMs accumulate in hypoxic regions of tumors in which hypoxia activates a pro-angiogenic program. The pro-angiogenic protein vascular endothelial growth factor (VEGF) and related molecules contribute to monocytes recruitment into primary tumours and in the metastatic niche [123,124]. In turn, the recruited leukocytes trigger an indirect pathway of angiogenesis, inducing secretion of pro-angiogenic factors [125–127]. TAMs increase the blood vessels density, increasing the quantity of circulating tumor cells and so metastasis.

Macrophages also mediate immunosuppression, involving MDSC, DCs and regulatory T cells. TAMs can express human leukocyte antigens (HLA) such as HLA-C, and HLA-G, HLA-E, or soluble forms that block the activation of a subsets of T cells and natural killer (NK) cells.

Finally, tumor infiltrating leukocytes induce the activation of the same transcription factors promoting their infiltration in tumor, resulting in an elevated production of inflammatory mediators that induce the CRI development.

2.3 Cancer-related inflammation and adaptive immunity

The malignant transformation involves the acquisition of epigenetic and genetic modifications that characterize the transformed cells. These modifications can be recognized by innate and adaptive immune mechanisms that protect the host against carcinogenesis. According to the theory of immune surveillance, the immune system surveys the body looking for tumor-associated molecules, eliminating many emerging tumors. The immune surveillance activity is one of the three phases of the immunoediting: elimination (immunesurveillance), equilibrium, and escape [128].

The innate immune response, which involves the inflammatory cascade, drives the beginning of adaptive immune responses. MyD88 is the adaptor protein of the Toll Like Receptor (TLR)

involved in innate immune responses. MyD88 is involved in oncogene-induced cell intrinsic inflammation and in cancer-associated extrinsic inflammation, and as such MyD88 contributes to skin, liver, pancreatic, and colon carcinogenesis, as well as sarcomagenesis. In contrast, in specific contexts MyD88 can promote the antitumor adaptive immunity [129].

In a clinically overt neoplasia the adaptive immune responses are suppressed by the activation of multiple pathways. The activation and differentiation of dendritic cells (which promote the adaptive immune responses), are blocked by signals (like IL-10) abundant in the tumour microenvironment. Moreover, regulatory T cells, which suppress both innate and adaptive immune responses, are often present in tumors. MDSCs, which as TAMs are strong suppressor of antitumour immunity, proliferate in tumour-bearing hosts [130,131].

Thus, in established tumours, in a high inflammatory context, there are multiple pathways that suppress effectively antitumour immunity.

2.4 Cancer-inhibitory inflammation

Although many studies showed that inflammation promotes tumor, there are also cases in which inflammation is not associated with cancer. For example, psoriasis does not enhance skin cancer thanks to the massive presence in skin of antitumoral M1 macrophages which balance the activity of the M2 TAMs. In certain contexts, inflammatory cells can destroy tumour cells. For example, TAMs in pancreatic tumours and in a subset of breast tumours, eosinophils in colon tumours and inflammatory tissue in bladder carcinoma are associated with better prognosis [132-134]. Furthermore, the treatment of various tumors with inflammatory cytokines like IFN-α, IL-2 and TNF-α induces significant benefits. [135-136].

A key regulator factor in the interplay between good inflammation and tumour promoting inflammation is NF-kb, which can regulate macrophage M1-M2 polarization. There are also

others factors involved in the regulation of this balance, like the tissue type. For example, in a skin tumour model, NF-κB over expression inhibits invasive epidermal neoplasia, whereas the interruption of NF-κB pathway inhibits the development of experimental liver and colon cancers. Moreover, studies of William Coley at the end of the nineteen century and recent studies have shown that bacterial infection in patients with tumour correlated with a better prognosis, as for example, the patients with bladder cancer treated with Mycobacterium bovis bacillus. These infections induce an adaptive immune response against tumor.

Experiments of treatment (chemotherapy and radiotherapy) of induced mouse breast cancers have shown that dying tumoral cells release HMGB1, a chromatin regulator protein and an inflammatory regulator, which drives a protective immune response. This regulation occurs in a TLR4 dependent-manner. Indeed, when tumours were developed in mutant TLR4 mice, the efficacy of therapy was reduced. In addition, human patients with breast mutated for TLR4 have a higher incidence of metastasis.

Thus, in determined conditions, the inflammatory antitumor response can prevail on the protumor inflammation.

3. The pentraxin superfamily

Innate immunity constitutes the first line of defence against microorganisms and is also important for the activation and regulation of adaptive immunity and for the maintenance of tissue repair and integrity [137].

Pentraxins are a superfamily of evolutionarily conserved proteins that, as component of the humoral arm of the innate immune system, play an important role in resistance against pathogens. Pentraxins are proteins of the acute phase of inflammation and are characterized by

a particular pentraxin domain of 200 amino acid in their C-terminal region, containing a "pentraxin signature" (HxCxS/TWxS, where x is any amino acid) (figure 11).

Based on this primary structure it is possible to distinguish short pentraxins, as the reactive-C protein (CRP) and serum amyloid P (SAP), and the long prentraxins, like PTX3. Pentraxins are conserved among the species during evolution from arachnids and insects to mammals.

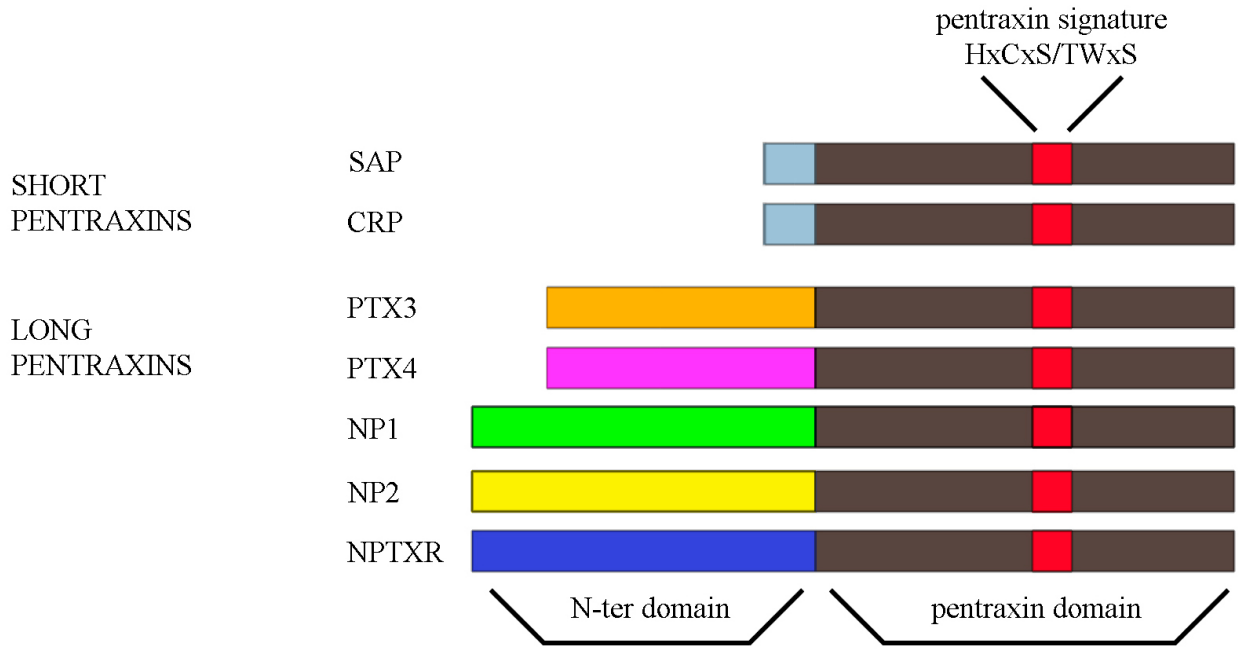


Figure and legend are from: Cecilia Garlanda, Barbara Bottazzi, Antonio Bastone, and Alberto Mantovani, Pentraxins at the Crossroads Between Innate Immunity, Inflammation, Matrix Deposition, and Female Fertility, Annu. Rev. Immunol. 2005

Figure 11: Short and long pentraxin structure. CRP and SAP belong to the short pentraxin subfamily and, like all pentraxins, are characterized by the presence of the pentraxin signature (HxCxS/TWxS, where x is any amino acid) in the 200 amino acid-long C-terminal domain. The Nterminal domain, typical of long pentraxins, like PTX3, is not conserved among the members of this subfamily.

3.1 The long pentraxin PTX3

3.1.1 Gene and protein structure and regulation

PTX3 is the archetype of the long pentraxin subfamily, characterized by a long N-terminal domain, associated with the C-terminal pentraxin-like domain. PTX3 was identified in the 1990's as a gene induced by cytokines in fibroblasts and endothelial cells [138,139]. Both the murine and the human *PTX3* genes are localized on chromosome 3 (3q25 in human) and are constituted by 3 exons. The first two exons code, respectively, for the leader peptide and for the N-terminal domain of the protein, whereas the last exon codes for the C-terminal pentraxin domain. In the proximal promoters of PTX3 genes, which are conserved among human and mouse, there are the binding sites for several transcriptional factors (like NF-kB, Pu1, AP-1 and SP1) (figure 12) [140] that regulate PTX3 expression in different contexts. In particular, AP-1 regulates the basal PTX3 transcription, while the response to the proinflammatory cytokines TNF-α and IL-1β is mediated by NF-κB [140,141]. In contrast, in alveolar epithelial cells TNF-α induces PTX3 production in a NF-kB independent manner and in, through the JNK pathway (142), while in endothelial cells high-density lipoproteins induce PTX3 production via PI3K/Akt activation [143]. The long pentraxin PTX3 is a multimeric glycoprotein characterized by a protomer of 381 amino acids including 17 amino acids for a signal peptide. The primary sequence of PTX3 is conserved during the evolution, suggesting the great importance of the functional role of PTX3 and of the structure/function relationships [144]. PTX3 has 203 amino acid in the C-terminal domain, homologous to the short pentraxins CRP and SAP, and has also a unique N-terminal domain, that is unrelated to any known protein. A three-dimensional model of PTX3 C-terminal domain has been built based on CRP and SAP crystallographic structures. This model showed that PTX3 pentraxin domain adopts a β-jelly roll topology. At Asn220 PTX3 has a single N-glycosylation site. This glycosidic site is occupied by complex-type

oligosaccharides, mainly sialylated and fucosylated biantennary sugars with just a minor fraction of tetra- and triantennary glycans [145]. PTX3 has a complex quaternary structure with protomer subunits assembled into oligomers of high order stabilized by disulfide bonds [146]. Site directed mutagenesis and mass spectrometry analyses of the recombinant protein show that human PTX3 is composed by octamers. The PTX3 N-terminal region has three cysteine residues at position 47, 49, and 103, that form the three inter-chain disulphide bonds that support the tetramer formation. Each octamer is made by two tetramers linked together through additional inter-chain bridges binding the two cysteines that are located at position 317 and 318 in the C-terminal domain. Small Angle X-ray Scattering (SAXS) and Electron Microscopy (EM) showed that the eight subunits of PTX3 protein fold into an elongated structure with a large and a small domain that are interconnected by a stalk region. The asymmetric shape of the molecule and this oligomerization state is unique amongst pentraxins.

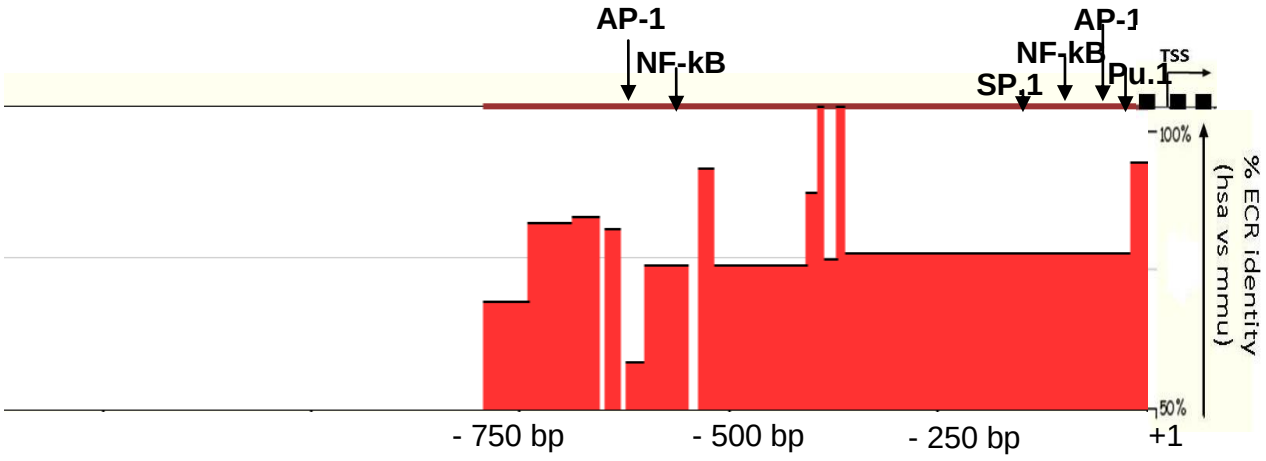


Figure 12: PTX3 proximal promoter transcription factor binding sites among human and mouse

The figure show the putative sites of binding of transcription factors on PTX3 promoter conserved among human and mouse.

PTX3 is produced by different cell types (i.e. monocytes, macrophages, dendritic cells, alveolar epithelial cells, uroepithelial cells, endothelial cells, chondrocytes, fibroblasts, and adipocytes) after stimulation by inflammatory signals such as TLR agonists, cytokines (e.g. TNF-α, IL1-β)

microbial moieties (e.g. OmpA, LPS, lipoarabinomannans) or microorganisms [147-149]. IFN-γ inhibits PTX3 production by monocytes, macrophages and dendritic cells, reducing both gene transcription and the transcript stability itself [150,151]. PTX3 induction by LPS is increased by IL-10. Enzymatically modified and oxidized low-density lipoproteins (ox-LDL) induce PTX3 production in human primary vascular smooth muscle cells (SMCs) and in endothelial cells (ECs). Glucocorticoid (GC) hormones also induce PTX3 production in non hematopoietic cells (like endothelial cells and fibroblasts), but inhibit PTX3 production in haematopoietic cells (like macrophages and dendritic cells) [152]. The GC receptor in haematopoietic cells interferes with the pathways of NF-κB and AP-1, whereas, in non-haematopoietic cells, it acts as a transcription factor.

In addition, PTX3 is stored in specific neutrophil granules, ready to be released in response to TLR agonists or microorganisms [153]. PTX3 messenger is not expressed in mature neutrophils but is confined to immature myeloid cells. PTX3 secreted partially localizes with neutrophil extracellular traps (NETs) that are generated upon neutrophil stimulation. Considering the high number of neutrophils in circulation, the PTX3 associated to neutrophils represents the major source of PTX3 covering a temporal window preceding the production dependent on gene expression. Most recently, PTX3 expression in uroepithelial cells was also reported. After stimulation with uropathogenic Escherichia coli (UPEC) PTX3 expression increased rapidly in a Toll-like receptor 4 (TLR4)- and MyD88-dependent manner [154].

3.2. PTX3 and complement

PTX3 can recognize and bind different ligands, as the component of the complement C1q, the main activator of the classical complement pathway [155]. The interaction between the globular head of C1q and PTX3 occurs in a calcium dependent-manner and leads either to inhibition or activation of the complement cascade. The interaction between plastic-immobilized PTX3 and

C1q induces the activation of the classical cascade of the complement and also the deposition of C3 and C4. Instead, the interaction between PTX3 and C1q in fluid phase inhibits the classical complement activation cascade through the competitive blocking of the relevant interaction sites. Furthermore, PTX3 binds at multiples sites the Factor H (FH), that is the main soluble regulator of complement alternative pathway, increasing the activity of FH itself and thus limiting an exacerbated activation of the complement cascade [156].

Finally, PTX3 interacts with C4b-binding protein (C4BP), which inhibits complement activation, helping the factor I in the inactivation and cleavage of C4b [157]. This interaction enhances C4BP recruitment on extracellular matrix and late apoptotic cells, suggesting that the C4BP recruitment by PTX3 may prevent an excessive local activation of the complement that would lead to inflammation and tissue damage.

PTX3 interacts with mannose-binding lectin (MBL), ficolin-1 and ficolin-2, that are three members of the lectin pathway. The complex MBL/PTX3 recruits C1q, enhances the phagocytosis of the pathogen and enhances also the deposition of C3 and C4 on Candida albicans. Similarly, immobilized PTX3 is able to trigger ficolin-1-dependent activation of the lectin complement pathway.

3.3 PTX3 in host defence

PTX3 binds a large spectrum of microorganisms, like fungi, viruses and bacteria. In particular, PTX3 binds *Pseudomonas aeruginosa* [158] *zymosan* and *Paracocc*idioides brasiliensis [159], conidia from Aspergillus fumigatus and Salmonella typhimurium [160], *Uropathogenic Escherichia Coli* (UPEC) [154], human and murine cytomegalovirus [161], and some strains of influenza virus [162].

Ptx3 -/- mice are more susceptible to invasive pulmonary aspergillosis, in fact they show higher mortality than wild-type mice. This phenotype is reverted by treatment with recombinant PTX3

that induces a protective Th1 response, as further demonstration that PTX3 has a role in the tuning of immune responses. The phagocytes that are deficient for PTX3 show a defective clearance and recognition of A. fumigatus. In accord to this, treatment with PTX3 reverses the phenotype, revealing the opsonic activity of PTX3 [163]. The phagocytosis of A. fumigatus conidia is increased by PTX3 through interaction with FcγRs [164,165]. This interaction triggers CR3 (CD11b/CD18) activation, promoting the phagocytosis of the pathogens opsonised by C3. Moreover, PTX3 facilitates also the recognition of P. aeruginosa by neutrophils. PTX3 maintains this prophagocytic effect in C1q-deficient mice but not in FcγR- and C3- deficient mice, suggesting that, the increase of phagocytosis of P. aeruginosa opsonised by PTX3 depends on the interplay between FcγRs and complement. PTX3 has therapeutic activity in a murine model of chronic P. aeruginosa lung infection that is a major cause of mortality and morbidity in cystic fibrosis patients. The bacterial lung colonization and the inflammatory response caused by the infection were reduced by treatment with recombinant PTX3.

Recently, PTX3 was identified as the first Pattern Recognition Molecules (PRM) essential for resistance against infection in the urinary tract induced by UPEC. In uroepithelial cells UPEC induce a rapid PTX3 expression through the pathway of Toll-like receptor 4 (TLR4)- and MyD88. In turn, PTX3 binds UPEC, enhancing their ingestion. In PTX3-deficient mice the defective recognition of UPEC is associated with elevated inflammation and tissue damage, proving the fundamental role of PTX3 in defence against urinary tract infections [154]. In human with urinary tract infections, PTX3 is in bladder sections and its systemic levels correlates with the clinical parameters of disease severity.

PTX3 can also amplify the inflammatory response, interacting with the Outer membrane protein A of K. pneumoniae (KpOmpA) in a complement dependent-manner. PTX3 binds both murine and human cytomegalovirus (MCMV), reducing infectivity in dendritic cells, in vitro. PTX3 binds also influenza virus (H3N2), of both human and murine, through an interaction between the viral haemagglutinin glycoprotein and the residue of sialic acid present on the glycosidic

moiety of PTX3. PTX3 has a range of antiviral activities, including inhibition of viral neuramidase activity, inhibition of hemagglutination and neutralization of virus infectivity. In contrast, PTX3 is inefficient against both pandemic and seasonal H1N1 influenza [166].

3.4 PTX3 and polymorphisms

The single-nucleotide polymorphisms (SNPs) of PTX3 gene are associated to different clinical conditions, above all in innate resistance to infections. PTX3 SNPs correlate with the risk of P. aeruginosa infections in cystic fibrosis patients, increased susceptibility to pulmonary tuberculosis, and with severe urinary tract infections [167,168]. Moreover, PTX3 polymorphisms correlated with protein levels and susceptibility to A. fumigatus infection in two cohorts of patients after bone marrow transplantation. PTX3 plasma levels are associated with three common genetic SNPs (rs2305619, rs3816527 and rs1840680), which may alter mRNA stability and consequently reduce PTX3 expression and decrease clearance and phagocytosis and of A. fumigatus [169]. The influence of PTX3 genetic variants was assessed also in acute myocardial infarction (AMI) [170]: different haplotypes of PTX3 correspond to different levels of PTX3 in plasma, which are correlated to all-cause death subsequent to AMI. Finally, genetic variants of PTX3 associated with primary graft dysfunction after lung transplantation are associated with increased PTX3 plasma levels [171].

3.5 PTX3 in inflammation

PTX3 specifically binds P-selectin through its N-linked glycosidic moiety and endogenous PTX3 produced and released from haematopoietic cells or administration of PTX3 triggers a negative loop feedback that prevents an excessive recruitment of neutrophils by P-selectin in a mouse models of pleurisy, acute lung injury (ALI), acute kidney injury and mesenteric

inflammation [172,173]. Indeed, PTX3 produced by haematopoietic cells works locally to regulate inflammation and reduce neutrophil recruitment.

A recent study has shown that PTX3 is involved in acute renal injury. In mice with renal ischemia-reperfusion injury, the lack of PTX3 enhanced postischemic acute kidney injury leading to tubular atrophy and interstitial fibrosis, whereas PTX3 recombinant injection prevented postischemic kidney injury [174]. PTX3 can modulate inflammation in atherosclerosis and acute myocardial infarction [175]. In mice, PTX3 is rapidly produced during acute myocardial ischemia and in PTX3-deficient mice, myocardial damages are increased, showing a cardio protective role for PTX3. In PTX3-deficient mice a higher deposition of C3 has been observed, maybe caused by the missing interaction between PTX3 and factor H, which avoids an exacerbated activation of the complement. Moreover, in apolipoprotein E-deficient mice with atherosclerosis, the lack of PTX3 is associated with elevated aortic lesions, increased number of macrophages within the plaque and an enhanced inflammation in the vascular wall.

In contrast to all the protective effects, in a mouse model of intestinal ischemia, the absence of PTX3 is accompanied by decreased tissue inflammation and lethality [176].

In the cumulus oophorus complex (COC) PTX3 is localized in the viscoelastic hyaluronan (HA)-rich matrix that forms around the oocyte [177]. PTX3 interacts with inter-α-trypsin inhibitor (IαI) and TSG-6, two molecules required for the incorporation into the matrix of the newly synthesized HA, an essential step for the organization and the stabilization of the matrix of cumuli. PTX3-deficient mice, because of the cumulus matrix instability, display a severe deficiency in female fertility.

3.6 PTX3 and cancer

In the context of cancer, PTX3 can be produced by tumor cells and stromal cells, such as leukocytes and endothelial cells. In vivo studies show that PTX3 can be protective against tumors.

PTX3 octamer has two binding sites for fibroblast growth factor 2 (FGF2) [178], which induces proliferation of endothelial cells, chemotaxis and in vivo neovascularization. Both FGF2 and PTX3, are produced by components of the vessel wall (as smooth muscle cells and endothelial cells) during inflammation. In murine melanoma B16-F10 cells, PTX3 inhibits FGF2-driven proliferation and stops the downstream FGFR signaling [179]. The overexpression of PTX3 in TRAMP-C2 cells (that are derived from a mouse model of prostate cancer) grafted in immunodeficient athymic male mice, inhibits their angiogenic and tumourigenic effects. In primary prostate human adenocarcinoma patients, PTX3 expression is lost in high-grade prostatic intraepithelial neoplasia [180]. Unpublished data of our laboratory showed that PTX3-deficiency, is associated with increased susceptibility to 7,12-dimethylbenz [α] anthracene/terephthalic acid (DMBA/TPA) and 3-Methylcholanthrene (3-MCA) in carcinogenesis (respectively epithelial and mesenchymal). In PTX3 knockout mice Complement activation, CCL2 production and tumor-promoting macrophage recruitment were improved.

Furthermore, in human esophageal squamous cell carcinoma, PTX3 is epigenetically down-regulated through promoter hypermethylation [181]. In contrast, in specific tumours PTX3 is a potential novel biomarker of cancer related inflammation, such as in gliomas [182] and lung carcinoma [183].

Togheter, all these data suggest that PTX3 is potentially involved in cancer development.

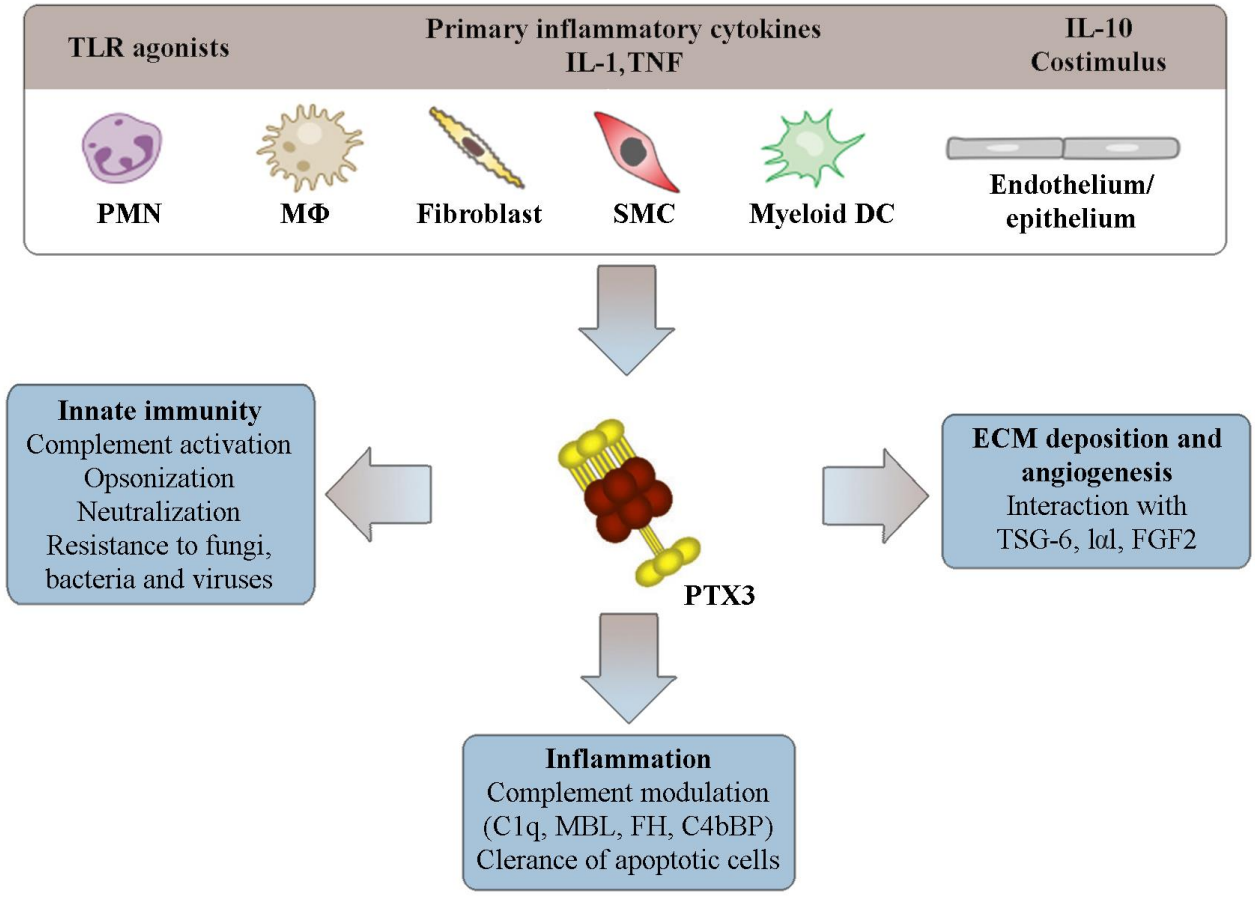


Figure and legend are from: Bottazzi, B., Doni, A., Garlanda, C. & Mantovani, A. An integrated view of humoral innate immunity: pentraxins as a paradigm. Annual review of immunology 2010.

Figure 13: Cellular sources and functions of the long pentraxin PTX3.

PTX3 is produced by phagocytes (PMN, MΦ, myeloid DC), fibroblasts, SMCs, ECs and, to a lesser extent, by epithelial and mesenchymal cells, upon stimulation with microbial moieties (TLR agonists) or primary inflammatory cytokines (IL-1, TNF); IL-10 acts as a costimulus for proinflammatory stimuli. Once released, PTX3 participates (i) in innate immune responses to fungi, bacteria, and viruses, through complement activation and regulation, microorganism opsonization, viral neutralization; (ii) in inflammatory responses, by modulating complement activation, by participating in the clearance of apoptotic cells, and possibly by other undefined mechanisms. PTX3 also participates in extracellular matrix deposition and angiogenesis.

Aims of the thesis

The long Pentraxin PTX3 has been shown to be rapidly induced upon inflammatory stimulation in different cell types. In particular, mononuclear phagocytes and endothelial cells have been shown to be major PTX3 producers in the context of acute inflammatory responses. In agreement, increased PTX3 levels are associated to the severity of different pathological conditions in humans, ranging from acute myocardial infarction to autoimmune diseases and infections. In animal models PTX3 deficiency leads to increased susceptibility to infections, excessive inflammation and tissue damage in sterile conditions and increased susceptibility to cancer.

After the fast induction in response to inflammation, PTX3 expression is rapidly deregulated. These data indicate that a fine tuning of PTX3 expression is essential in innate responses to pathogens and to keep under control PTX3-dependent modulation of the inflammatory response in sterile tissue damage, including cancer.

Thus, the general aim of this study was to gain insight into the genetic and molecular mechanisms at the base of regulation of PTX3 expression in basal condition, during inflammatory responses and in cancer.

Specific aims were:

Aim 1- Identification of the regulatory elements involved in the modulation of PTX3 expression both in basal and in inflammatory conditions and gain insight into their mechanisms of action.

Aim 2- Investigation of the potential regulatory role of miRNAs in the modulation of PTX3 expression during inflammatory responses acting by direct and indirect mechanisms.

Aim 3- Analysis of the regulation of PTX3 expression by epigenetics modifications in cancer.

MATERIALS AND METHODS

Prediction of cis-regulatory regions.

The bioinformatics tool Genomic Regions Enrichment of Annotations tools (GREAT) assigns biological meaning to a set of non-coding genomic regions by analyzing the annotations of the nearby genes. Thus, it identifies the cis functions of sets of genomic regions: putative enhancer.

Analysis of the conserved regions among the species.

The ECR Browser analyzes Evolutionary Conserved Regions (ECRs) in genomes among several sequenced species.

The browser aligning the sequence of the one selected genome with others in a pairwise fashion, creating a conservation profile, which can be graphically displayed for any locus of the selected genome. ECRs are identified as regions of high sequence identity against a neutrally evolving background. By scanning the pairwise alignments, the browser detects sequence elements of significant length that are conserved above a certain level of sequence identity between the two genomes and highlights them as ECRs.

Cell purification, culture and transfection.

Human studies were approved by the ethical committee of Humanitas Clinical and Research Center (Milan, Italy). Human monocytes were obtained from buffy coats of healthy donors by two-step gradient centrifugation, first by Histopaque-1077 (Sigma-Aldrich) and then by Percoll (GE Healthcare). Residual T and B cells were removed from monocyte fraction by plastic adherence. Monocytes were cultured in RPMI-1640 medium supplemented with 10% FBS and they were differentiated to macrophages by adding 100 ng/mL M-CSF. Macrophages were stimulated or not with 100 ng/mL LPS for indicated times.

The 8387 human fibrosarcoma cell line (ATCC) were cultured at 37ºC with 5% CO_2 in RPMI supplemented with 10% FBS, 2 mM L-glutamine, and antibiotics (all reagents from Lonza). The HEK293T cell line constitutively expressing the SV40 large T antigen (ATCC) was cultured at 37ºC with 5% CO_2 in DMEM (Lonza) supplemented with 10% FBS, 2 mM L-glutamine, and antibiotics.

For mimic transfection, 8 x 10^4 8387 cells were plated in complete medium without antibiotics in 24-well tissue culture plates and transfected 24 h later by lipofectamine 2000 reagent (Invitrogen). miR mimics and respective scramble controls were added to each well to 100 nM final concentration.

Colorectal cancers (CRC) can be genetically classified based on mutations of mismatch repair genes, which are associated the microsatellite instability. Thus CRCs may be distinguished in microsatellite stable (MSS) or microsatellite instable (MSI). Six human CRC cell lines were utilized in this study, 3 CRC MSS cells (HT29, SW480, SW620) and 3 CRC MSI cells (RKO, SW620, LOVO) (from American type culture collection (ATCC). As the normal control for RNA expression in CRC cells, we used Human Colonic Epithelial Cell line (HCoEpiC) (from ScienceCell Research Laboratories). The CRC cell lines were cultured in RPMI medium supplemented with 10% fetal bovine serum and 1% ultraglutamine at 37°C in a humidified 5% CO_2 incubator. The HCoEpiC cell lines were cultured in Colonic Epithelial Cell Medium (CoEpiCM, Cat. No. 2951). When specified, cells were treated with the epigenetic modifier 5-aza-2'-deoxycytidine (5-Aza-dC, 15 μmol/mL) (A3656-5MG Sigma-Aldrich) for 72 h and with Trichostatin A (TSA, 150nm/mL) (T8552-1MG Sigma-Aldrich) for 48 h. The medium containing 5-Aza-dC was renewed every 24 h. Then cells were washed with PBS and stimulated with TNFα 20 ng/mL or IL-1β 20 ng/mL for 4 h or 24 h to analyse PTX3 mRNA and protein expression, respectively.

Real time RT-PCR (RT-qPCR) analysis.

Total RNA was extracted using the TRIzol method according to the manufacturer's instructions (Ambion). RNA purity and concentration were evaluated using NanoDrop 1000 spectrophotometer (Thermo Scientific). 1 μg of total RNA from each sample was reverse transcribed using the high capacity cDNA Reverse Transcription kit (Applied Biosystems), according to the manufacturer's protocol. *PTX3* and *Erp18* gene expression was quantified by RT-qPCR performed in triplicate in the presence of the SYBR green PCR master mix (Applied Biosystems) and specific oligonucleotides

(PTX3: FW:5'-CGAAATAGACAATGGACTCCATCC-3'; REV:5'-GCAGGCGCACGGCGT-3'). RT-qPCR data were analysed with SDS 2.2.2 software (Applied Biosystems) and normalized on the expression of GAPDH expression (FW: 5'-AACAGCCTCAAGATCATCAGC-3'; REV: 5'-GGATGATGTTCTGGAGAGCC-3'). According to the manufacturer instructions, TaqMan microRNA Human Assays were used to synthesize cDNA from 45 ng RNA using individual miR-specific RT primers and to amplify by qPCR each generated cDNA with sequence-specific primers in triplicate. The ubiquitous small nuclear U6, which showed stable expression across the time and the conditions tested, was chosen as the internal control for all of the experiments. RT-qPCR reaction was conducted using the 7900HT Fast Real-Time PCR System (Applied Biosystems). Thermal cycler conditions were the following for mRNA analysis: 95 °C for 20 s, 40 cycles of 95 °C for 1 s and 60 °C for 20 s. An initial step at 55°C for 2 min was added for miR expression analysis.

ELISA assay.

PTX3 protein released by 8387 cells in cell-free supernatants was quantified by home-made-ELISA. In brief, 96-well ELISA plates (Thermo Scientific) were coated with 100 μg of anti-PTX3 mAb MNB4 and incubated ON at 4°C. Nonspecific binding sites were saturated with 250 μL of 5% dry milk (2 h at RT) prior the addition of purified human recombinant PTX3 standards

(from 75 pg/mL to 2.4 ng/mL) and unknown samples diluted in PBS and 2% BSA (Sigma-Aldrich). After 2 h at 37°C, 100 ng biotin-conjugated rabbit polyclonal anti-PTX3 was added to each well. Plates were incubated for 1 h at 37°C and streptavidin-peroxidase (Biospa) was added, followed by the addition of the TMB liquid substrate system (Sigma-Aldrich). After each step, plates were extensively washed with PBS containing 0.05% Tween-20 (Merck Millipore), also used for reagent dilution.

Chromatin immunoprecipitation.

Briefly, $12x10^6$ cells were used for each ChIP. Cells were cross-linked for 10 minutes at room temperature using 1% formaldehyde. Cross-linking was quenched by adding glycine to a final concentration of 0.125M. The cells were then collected, resuspended in lysis buffer (5mM PIPES pH 8, 85mM KCl, 0.5% NP40 and protease inhibitors), and incubated on ice for 30 min. before proceeding with sonication to generate 200−400bp fragments. The efficiency of sonication was assessed with agarose gel electrophoresis. Chromatin samples were pre-cleared for 1 hour with protein-G beads and then immunoprecipitated overnight at 4°C with 5 μg of the following specific antibodies: anti-H3 (Abcam, ab1791), anti-H3K27ac (Abcam code, ab4729), anti-H3K4me3 (me, methylation; Active Motif code 39159), anti-H3K4me1 (Abcam code, ab8895), anti-H3K27me3 (Millipore-Upstate code, 07–449) and anti-NFκB p65 (RelA) (Millipore-Upstate code, 17–10060), anti-Pol II (N-20) (Santa Cruz biochemistry sc-899), anti-EZH2 (Millipore), anti SUZ12 (Millipore) and rabbit IgG (Millipore; 12–370) was used as negative control. After incubation, the immunocomplexes were bound to protein-G beads for 2 hours and subsequently washed with low-salt wash buffer (0.1% SDS, 2mM EDTA, 20mM Tris HCl pH8, 1% Triton X-100, 150mM NaCl and protease inhibitors), high-salt wash buffer (0.1% SDS, 2 mM EDTA, 20mM Tris HCl pH8, 1% Triton x-100, 500mM NaCl and protease inhibitors), and TE buffer. Immunocomplexes were then eluted in elution buffer (1% SDS, 100mM $NaHCO_3$) and cross-

linking reverted overnight at 65°C. Samples were then treated with proteinase K, and then the DNA was purified with QIAquick PCR purification kit (Qiagen).

Quantitative real-time PCR was performed in triplicate by using the following primers: *PTX3* Exon 2 (region 1): forward 5'-GAG CTA CAT AAC CGG CGA-3', reverse 5'-GCC AAG TTG CAG CCC GTT CC-3'; *PTX3* exon 2 (region 2): forward 5'-GGA ACG GGC TGC AAC TTG G-3', reverse 5'-GTC CCA TTC CGA GTG CTC C-3'; *PTX3* exon 2 (region 3): forward 5'-GCA CTC GGA ATG GGA CAA GC-3', reverse 5'-CGC GCA CGG CCT CGC CAG-3'; *PTX3* exon 2 (region 4): forward 5'-CTG GCG AGG CCG TGC GCG-3', reverse 5'-CCT CTA GCA CCG CGG CCA G-3'; *PTX3* exon 2 (region 5): forward 5'-GGG CGC GCC CTG GCC GCG-3', reverse 5'-GTG GGA GGT CCC GGC CCC-3'; *PTX3* exon 2 (region 6): forward 5'-GGG GAA GCT TTC ATG GGA AGC-3', reverse 5'-CCG CCT TGT ATG GTG TGC CTG-3'; *PTX3* exon 2 (region 7): forward 5'-CAC AGT CTT GAA ATA GAC GTC-3', reverse 5'-GAA GAC GTG GAA ATC CCT AAC-3'; *PTX3* promoter (region 1): forward 5'-TGT CAT AGG CCG GGT GAG G-3', reverse 5'-CCG CCA CAC CCA GTT AAT-3'; *PTX3* promoter (transcription factors binding site (TFBS) region): forward 5'-TGG CAC TGC GGT AAC GGG A-3', reverse 5'-CCT CCA ATT AAT CTG ACT GC-3'; PTX3 first enhancer: forward 5'-TCTGTGATTTGCCCAAGAGA -3', reverse 5'- GACAAGAAAAGAGGCCCTGG-3'; PTX3-500 bp before first enhancer: forward 5'-GCGGTCTTTTCCCTTGGTCC-3', reverse 5'-CCTCAGACCCAGGAAGAGTC-3'; PTX3-500 bp after first enhancer: forward 5'-CACAGGCGTTCATTGTC-3', reverse 3'AACTGGGAACACGTGCCAC-3.

Signals obtained from the ChIP samples were normalized on signals obtained from corresponding input samples, according to the formula 100 × 2^ (input Ct − sample Ct). Binding is expressed as percentage of enrichment respect to IgG.

Construct generation and luciferase reporter assay.

The PTX3 3'-UTR was amplified from the human PTX3 coding sequence (CDS), previously cloned in pBlueScript vector, by PCR with the M13 primer (5'-TGTAAAACGACGGCCAGT-3') and the following primer 5'-GAACTAGTATGTTGTGAAACTCCACTTG-3' containing a SpeI restriction site (underlined), excised by SpeI-XhoI digestion and subcloned in psiCHECK-2 vector (Promega) (Rluc-PTX3). Site-directed mutagenesis reactions were conducted using the QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies), in accordance with manufacturer's instructions. The following constructs were generated with mutated (underlined region) oligonucleotides:

Rluc-mut-9-PTX3 (FW: 5'-CTCCACTTGAAGCGGGGGAAAGAAACTCACACT-3';

REV: 5'-AGTGTGAGTTTCTTTCCCCCGCTTCAAGTGGAG-3'),

Rluc-mut-29-PTX3 (FW: 5'-CTAGACTTTATGCCAGCCGCCTTTCAGTTTAATGC-3',

REV: 5'-GCATTAAACTGAAAGGCGGCTGGCATAAAGTCTAG-3'),

Rluc-mut-181 (FW:5'-GTCAACTTGTCATTGCGCCGAAAGAATGAAACCTTC-3';

REV: 5'-GAAGGTTTCATTCTTTCGGCGCAATGACAAGTTGAC-3'),

All clones were sequenced and subsequently used in the luciferase reporter assay. pGV-B2 plasmid, expressing *firefly* luciferase under the control of human *PTX3* promoter (Accession number X97748), was obtained as previously reported. pRL-TK vector, that expresses *renilla* luciferase gene under an eukaryotic constitutive promoter (HSV-thymidine kinase promoter), was used as internal control.

The NF-κB luciferase reporter vector from BPS Bioscience (60614) contains a firefly luciferase gene under the control of multimerized NF-kB responsive element located upstream of a minimal promoter. The NF-kB reporter is premixed with constitutively-expressing

Renilla luciferase vector, whichserves as an internal control for transfection efficiency.

To study the direct interaction of miR and 3'-UTR with the luciferase reporter assay, 8 x 10^4 HEK293T cells were plated in 24-well plates in DMEM supplemented with 10% FBS and 2 mM

L-glutamine and co-transfected 24 h later using lipofectamine 2000 (Invitrogen) with 100 ng psiCHECK-2 (Rluc constructs) or pEZX-MT01 vectors (Luc constructs) and miR mimics or miR negative control (scramble; Ambion) at a final concentration of 100 nM. HEK293T cells were co-transfected with 300 ng pGV-B2 and 100 ng of the control vector pRL-TK, and 100 nM miR mimics or scramble and then stimulated or not with 20 ng/mL TNFα.

HEK293T cells were also co-transfected with 100 ng of NF-kb reporter vector and 100 nM miR mimics or scramble and then stimulated or not with 20 ng/mL TNFα. After 24 h, cells were lysed and both *firefly* and *renilla* luciferase activities were determined using the Dual-Glo Luciferase Assay System (Promega) and quantified using a MultiDetection Microplate Reader Synergy 2 luminometer (BioTek Instruments).

The use of psiCHECK-2 and pEZX-MT01 vector enabled the detection of changes in the expression of the target gene (3'-UTR) fused to a primary reporter gene (*renilla* luciferase, Rluc, for psiCHECK-2 vector and *firefly* luciferase, Luc, for pEZX-MT01 vector). The second reporter gene (Luc for psiCHECK-2 vector and Rluc for pEZX-MT01 vector) was used as an internal control, allowing the normalization of primary reporter gene expression.

Results obtained from the co-transfection of 3'-UTR constructs and mimics were expressed as mean ± SEM of the ratio between primary luciferase and control luciferase activities adjusted to 1.

Generation of PTX3-inflammatory molecular network

PTX3-inflammatory gene network has been generated and graphically visualized with Ingenuity Pathway Analysis (www.ingenuity.com), according to IPA predefined settings. The IPA Ingenuity software uses a dataset of experimental publish data to built the interaction network of the selected protein, calculating the P value associated to the Fisher exact test. A list of predicted target genes for each miR was derived from microrna.org database, taking into account both conserved and poorly conserved target sites.

Immunoprecipitation of Ago2-bound RNA species (RIP).

Briefly ,5 to 10 x 106 of cells for each experimental point were pelletted, washed with PBS-/- 1x and lysed in 300 μl of lysis buffer (150 mM KCL, 25 mM Tris PH 7.4, 5 mM EDTA, 0,5% NP40, 5 mM DTT, protease inhibitor cocktails (Roche), 100 U/ml Superase-In (Ambion). The cells were lived on ice for 30 min and then centrifuged 14000 rpm for 30 min at 4°C. The supernatants were incubated with Dynabeads Protein G magnetic beads (Merck Millipore) conjugated with anti-Ago2 (EIF2C2 monoclonal antibody, clone 2E12-1C9; Abnova) or isotype IgG1k control antibodies (Abnova). After the immunoprecipitations, we kept the flow-through which contains the Ago2 unbound fraction, labeled "left over ip". The beads with the Ago2 bound fraction were washed 3 times with the lyses buffer, before the direct RNA extraction with the TRizol. Results were expressed as fold enrichment relative to IgG1k-IP control samples. The miR/mRNA enrichment in the RNA-induced silencing complex was calculated according to the formula $2^{-(CtAgo - CtIgG)}$ and results were expressed as fold enrichment relative to IgG-IP control samples.

PTX3 expression profiles in cancer

Using Oncomine gene expression browser (www.oncomine.org) to check the level of PTX3 mRNA in several human healthy tissue and cancer types.

Tissue samples

Patients: Paraffin-embedded tissues from oncologic Caucasian patients, who underwent resection surgery at Humanitas Clinical and Research Center, were examined for *PTX3* gene epigenetic modification. The study included the following cases: 3 angiomyosarcomas, 5 leiomyosarcomas, 5 synovial sarcomas, 5 solitary fibrous tumors, 5 chordomas, 5 gastrointestinal stromal tumors, 3 desmoid tumors, 6 squamous cell carcinoma, 5 high grade adenomas, 10 stage I colorectal cancer (CRC), 10 stage II CRC, 10 stage III CRC and 10 stage IV CRC. The

Institutional Review Board of the Humanitas Clinical and Research Center, Rozzano, Italy, approved this study (ICH-99/09).

Adenoma laser microdissection: Paraffin-embedded, hematoxylin and eosin (H&E)-stained slides were reviewed by a pathologist (M.N.). Six sections 5 μm thick were cut from selected formalin-fixed, paraffin-embedded blocks and mounted on PEN membrane glass slides (LCM tissue slides, Life Technologies, Monza Italy). One section from each sample was stained with H&E to select areas of high-grade adenoma for DNA purification. The remaining sections were used to collect adenoma tissue by laser capture microdissection (Leica LMD 7000).

Methylated CpG Island Recovery Assay (MIRA).

Genomic DNA from cell cultures or paraffin-embedded tissues was purified by QIAamp DNA Midi kit (QIAGEN) and fragmented by sonication to about 400 bp average sizes as verified on agarose gel. Enrichment of the methylated double-stranded DNA fraction was performed with the MethylCollector Ultra Kit (Active Motif), according to manufacturer's instructions. Eluted DNA was purified with QIAquick PCR purification kit (Qiagen). Quantitative real-time PCR was performed in three promoter regions containing at least 5 CpG, and on the CpG island divided in 7 about 120 bp regions. Quantitative real-time PCR was performed in triplicate by using the following primers: *PTX3* Exon 2 (region 1): forward 5'-GAG CTA CAT AAC CGG CGA-3', reverse 5'-GCC AAG TTG CAG CCC GTT CC-3'; *PTX3* exon 2 (region 2): forward 5'-GGA ACG GGC TGC AAC TTG G-3', reverse 5'-GTC CCA TTC CGA GTG CTC C-3'; *PTX3* exon 2 (region 3): forward 5'-GCA CTC GGA ATG GGA CAA GC-3', reverse 5'-CGC GCA CGG CCT CGC CAG-3'; *PTX3* exon 2 (region 4): forward 5'-CTG GCG AGG CCG TGC GCG-3', reverse 5'-CCT CTA GCA CCG CGG CCA G-3'; *PTX3* exon 2 (region 5): forward 5'-GGG CGC GCC CTG GCC GCG-3', reverse 5'-GTG GGA GGT CCC GGC CCC-3'; *PTX3* exon 2 (region 6): forward 5'-GGG GAA GCT TTC ATG GGA AGC-3', reverse 5'-CCG CCT TGT ATG GTG TGC CTG-3'; *PTX3* exon 2 (region 7): forward 5'-CAC AGT CTT GAA ATA

GAC GTC-3', reverse 5'-GAA GAC GTG GAA ATC CCT AAC-3'; *PTX3* promoter (region 1): forward 5'-TGT CAT AGG CCG GGT GAG G-3', reverse 5'-CCG CCA CAC CCA GTT AAT-3'; *PTX3* promoter (region 2): forward 5'- TCG AGA CCA GCC TGG CTA A-3', reverse 5'-GCA ATG GTG CTC CCG CAT C-3' ; *PTX3* promoter (transcription factors binding site (TFBS) region): forward 5'-TGG CAC TGC GGT AAC GGG A-3', reverse 5'-CCT CCA ATT AAT CTG ACT GC-3'. PTX3 first enhancer: forward 5'-TCTGTGATTTGCCCAAGAGA -3', reverse 5'- GACAAGAAAAGAGGCCCTGG-3'.

For all samples, MIRA-enriched DNA was compared with input DNA, according to the formula $100\times2^{(\text{input Ct} - \text{sample Ct})}$, normalized to the value of the positive control provided with MIRA kit (NBR2) and expressed as percentage of enrichment.

Statistical analysis

Data were expressed as means ± SEM of N experiments or donors, as specified. Dunnett's *post-hoc* test following a one-way ANOVA or Student's *t*-test were used, as specified, to determine statistical changes. $p \leq 0.05$ were considered significant. Statistics were calculated with GraphPad Prism version 5 (GraphPad Software).

Statistical analysis was performed using the Student's *t* test and the $\chi 2$ test; a value of $P < 0.05$ was considered to be statistically significant.

Immunohistochemistry.

Four-µm consecutive sections were cut from the CRC Paraffin-embedded tissues and used for PTX3 immunohistochemistry. Sections were dewaxed in Xilol or Bioclear, then they were hydrated with rapid wash in Ethanol 100% , Ethanol 90%, Ethanol 70% and then in H_2O. Antigens were unmasked in microwave oven 2 step for 5 min at 800MW in EDTA pH 8.00 buffer 0.25 mM. Then the endogenous peroxidase was blocked for 5 min with a Peroxidized 1

(Biocare Medical) ready to use. Unspecific sites were blocked with Background Sniper (#BS966G Biocare Medical) for 15 min and tissues were incubated for two hours with Rabbit polyclonal antibody anti Human PTX3 purified (rabbit polyclonal IgG) in in *DaVinci Green Diluent* (#PD900H Biocare Medical). MACH 4 Universal HRP-Polymer Detection (cod: M4U534, Biocare Medical) was used as secondary antibody for 30 minutes. After washing, slides were developed with DAB (3,3'-diaminobenzidine) (Biocare Medical) and counterstained with Hematoxilyn. Tissues were dehydrated with ethanol, mounted with Eukitt and analysed with an Olympus BX61 virtual slide scanning system.

Result

1. Characterization of PTX3 regulatory elements in basal conditions and in response to inflammatory stimuli

1.1 Bioinformatics analysis revealed 2 enhancers potentially involved in the regulation of the long pentraxin PTX3.

In order to identify regulatory elements, such as miRNAs and enhancers, that could potentially modulate PTX3 gene expression, we first used bioinformatics tools to screen the whole PTX3 sequence. As shown in Figure 14, PTX3 has a promoter upstream of 3 coding sequences and a CpG island localizing with the second. We checked also the microRNAs that could target PTX3 3'UTR and identified 8 different miRNAs: has-miR-9, has-miR-29a/b/c and has-miR-181a/b/c/d. We identified enhancer regions using raw data from Chip-seq analysis of histone modifications, found on Geo profiles: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE36401. We analysed three samples of human normal colon mucosa and 3 samples of human colon cancer. Using the Genomic Regions Enrichment of Annotations Tool (GREAT), that assessed the functional significance of several enhancers, we found 2 putative enhancers that could regulate the PTX3 gene. As shown in figure 15, these putative genetic elements are located on chr3:156893652-156894644 (the first enhancer) 230 kb upstream the gene promoter region and spanning the second exon of the PTX3 gene (hg19 chr3:156,893,632-156,894,644) (the second enhancer), respectively.

We then verified the conservation of these sequences among species, using ECR browser and we observed that the exonic enhancer was highly conserved (more than 70%), whereas the first enhancer was less conserved (50%) (Figure 15).

Since DNA methylation is involved in regulating the activity of enhancers, we also wondered whether CpG islands were present in these genetic elements. In order to do this, we used Genome Browser and the softwares Epiginomes and Methyl Primer Express v1.0, which allowed us to identify CpG sites and calculate their enrichment in the regions of interest.

The analysis (Figure 15) showed that the exonic enhancer localized in a CpG island, whereas both the promoter and the first enhancer are CpG-poor sites. We also observed that both these regulatory elements shared putative binding sites for RNA Polymerase II and for transcription factors that characterize enhancers, including P300.

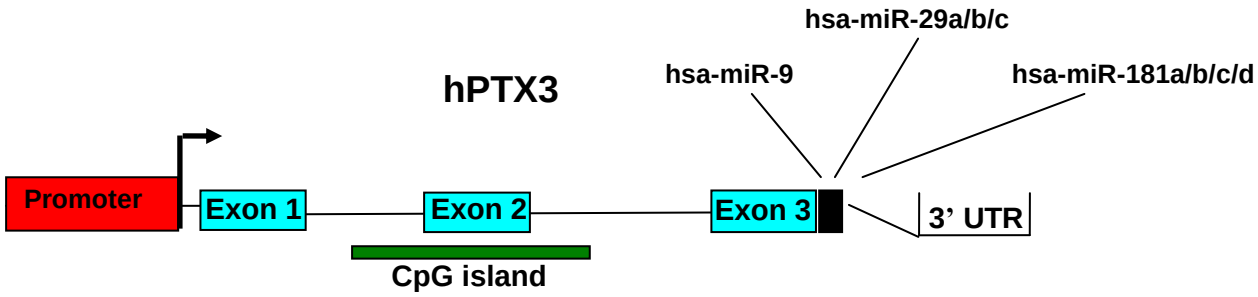


Figure 14: Human Long Pentraxin PTX3 coding sequences. PTX3 mRNA is organized in 3 exons (light blue) and 2 introns. PTX3 second exon localized in CpG island 557 bp long. Human PTX3 3' UTR is the putative target of several miRNAs, including miR9, miR29 family and miR 181 family.

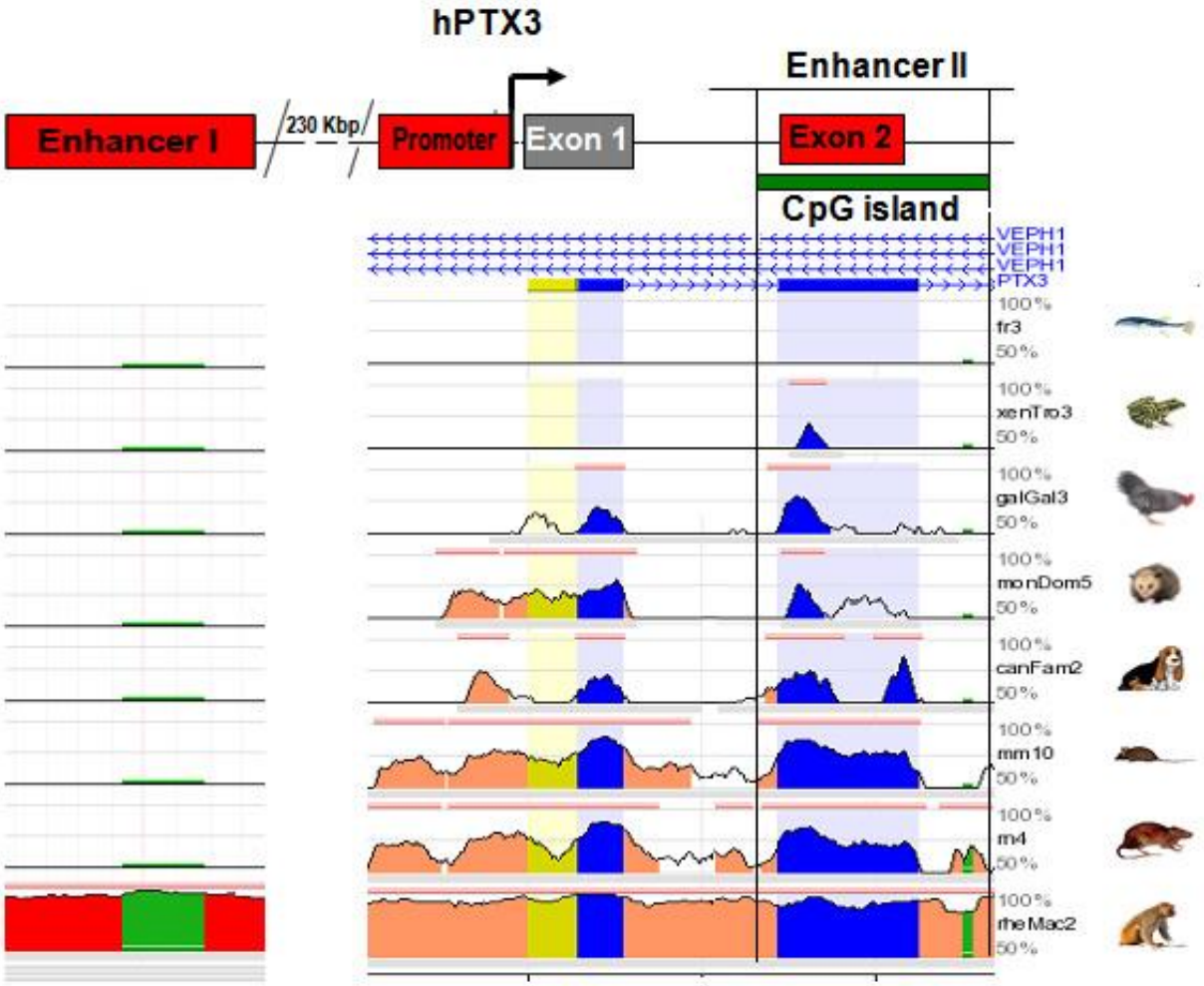


Figure 15: PTX3 putative enhancers are conserved among species. Analysis of genome conservation among species of the human PTX3 gene and putative enhancer through ECR browser. The conservation rate is calculated by the browser comparing the sequence of the PTX3 human gene among species: fugu, frog, chicken, opossum, dog, mouse, rat and Rhesus macaque, respectively.

1.2 PTX3 enhancers are activated by inflammatory stimuli in the 8387 cell line.

To gain insight into the activation state of the two enhancers associated to PTX3,we first checked the mRNA transcription levels of the gene and to determine the peak of maximum expression, which is dependent on the activity of regulatory elements.

We analyzed PTX3 expression and production in the 8387 human fibroblast cell line, that produces a detectable amount of PTX3 even in basal conditions, as shown by previous results in our laboratory. As shown in figure 16, PTX3 expression increased in response to TNFα and IL-1β inflammatory stimuli: PTX3 mRNA was highly expressed at 4h and it decreased at 8h and 24h. We observed the highest level of PTX3 mRNA expression 4 hours after treatment with TNFα, whereas the protein level reached a peak 24 hours upon treatment.

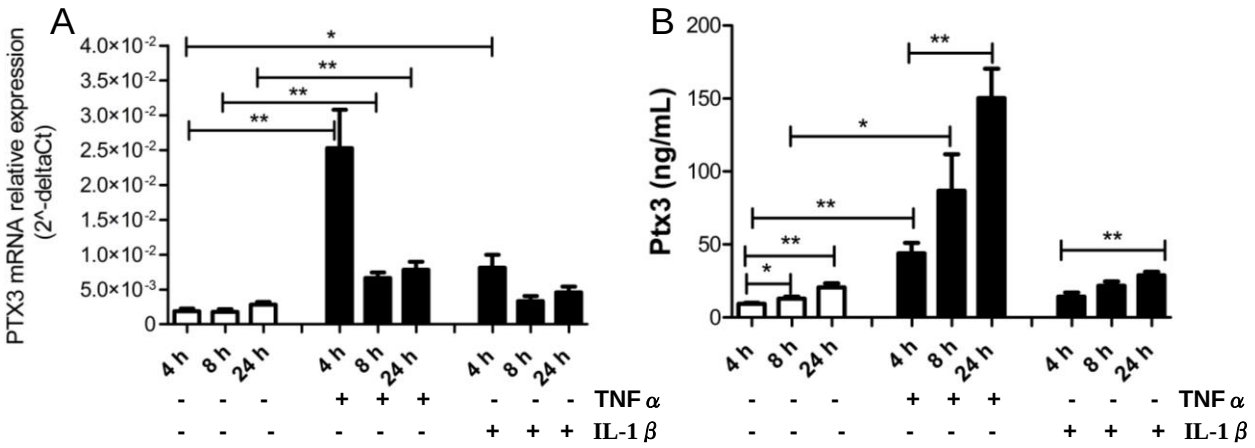


Figure 16: PTX3 mRNA expression and protein production in the 8387 cell line.

A-B) Kinetic analysis by RT-qPCR of PTX3 mRNA expression (A) and protein production (B) in 8387 cells as measured by ELISA in the cell supernatant, in normal conditions and after 20 ng/ml TNFα and IL-1β treatment. Results are expressed as $2^{\Lambda-\Lambda Ct}$ (A) and as ng/mL (B) and as mean ± SEM (N = 4 experiments). *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$; Student's *t*-test.

To validate the activity of the putative enhancers and to characterize their functional role, we performed Chromatin Immune Precipitation (ChIP) assays using antibodies specific for the histone modifications that characterize active enhancers (H3K4me1, H3K27Ac) and inactive enhancers (H3K27me3). As positive control, we used antibodies specific for Histone 3 (H3), which is constitutively expressed. As negative control, we also checked the levels of histone modifications in the regions upstream and downstream the sites of interest. Regarding the first enhancer, we analysed 3 sites: a central region, which is the most conserved among species

(Figure 2) and 2 other regions 500 bp away from it. Concerning the second enhancer, we analysed 7 fragments. After cell stimulation with an inflammatory cytokine, the two regulatory elements switched from an inactive to an active state and this correlates with an higher PTX3 expression. The results related to the first putative regulatory element indicated that the central region is the effective enhancer, since it showed high levels of active markers and low levels of repressor markers (figure 17). Thus, in the following analysis we did not further investigate the adjacent regions. The putative regulatory elements were functional enhancers in the 8387 cell line.

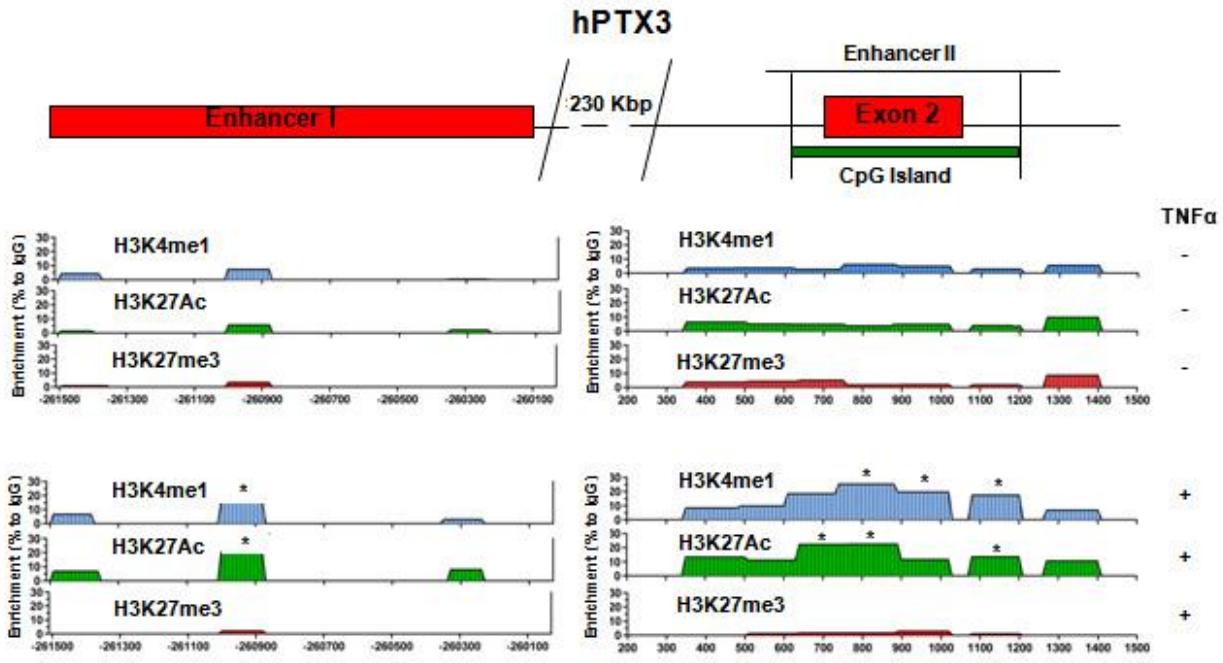


Figure 17. Chromatin Immunoprecipitation of histone modifications revealed that PTX3 have 2 enhancers.

ChIP assay in the 8387 cell line in basal condition and 4h after stimulation with TNFα using antibodies recognizing H3K4me1, H3K27Ac and H3K27me3 histone modifications. Results are expressed as percentage of enrichment to IgG and as a mean ± SEM (N = 3 experiments) of values obtained according to the formula 100 × 2^ (input Ct − sample Ct) and then normalized on IgG. *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$; Student's *t*-test. Each region analyzed with RT-qPCR is 150 bp long.

1.3 The PTX3 promoter is active both in basal and in inflammatory condition in the 8387 cell line.

Given that enhancers regulate gene expression acting on the promoter, we then analysed the PTX3 promoter activity in the 8387 cell line both in basal condition and upon treatment with TNFα. Histone signature of promoters consists of modifications associated with both transcriptional activation (H3K4me3, H3K9Ac and H3K27Ac) and repression (H3K27me3), which are dependent on the Polycomb group complex. We analyzed 2 different regions: the first, called P1, that is located 500 bp before the transcription starting site (TSS) and the transcription factor binding site (TFBS) region, that is a putative binding site for several transcription factors and that is 20 bases upstream the TSS.

PTX3 promoter (figure 18) was demonstrated to be active even in basal condition, but upon stimulation with TNFα, we observed a significant increase in the activation of the TFBS, which is the closest to the TSS and the most enriched in nucleosomes.

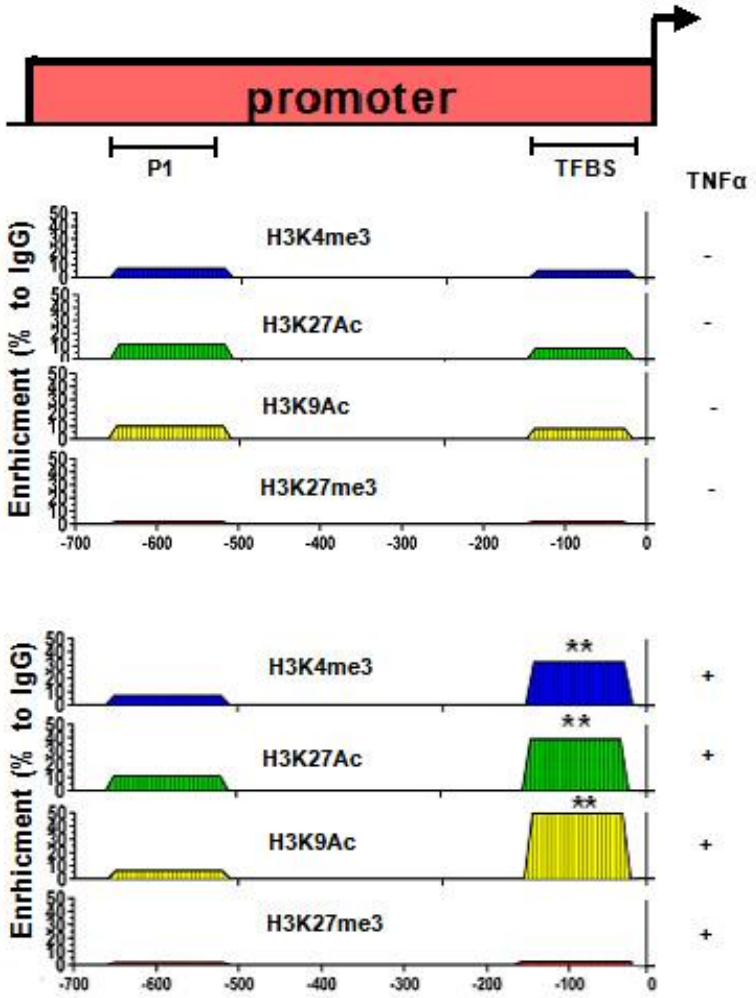


Figure 18. PTX3 promoter activity increased after treatment with TNFα in the 8387 cell line.

ChIP assay in the 8387 cell line in basal condition and 4h after stimulation with TNFα using antibodies recognizing H3K4me3, H3K27Ac, H3K9Ac and H3K27me3 histone modifications. Results are expressed as percentage of enrichment to IgG and as a mean ± SEM (N = 3 experiments) of values obtained according to the formula 100 × 2^ (input Ct − sample Ct) and then normalized on IgG. *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$; Student's *t*-test. Each region analyzed with RT-qPCR is 150 bp long.

1.4 Inflammatory cytokines enhance PTX3 expression improving RNA Polymerase II activity in a NF-kB dependent manner.

The expression of PTX3, which is a protein of the acute phase of inflammation, is regulated by inflammatory transcription factors, including NF-kB in response to inflammatory stimuli, such as TNFα. To verify whether PTX3 enhancer activation was mediated by this factor, we performed a Chip assay for the NF-kB active subunit p65 in 8387 cells, both in basal conditions and 4 hours after treatment with TNFα (figure 19). As negative control, we used the exon 2 of the PTX3 gene, which lacks putative binding sites for this transcription factor. The result show that the inflammatory cytokine TNFα induced the binding of NF-kB to both the PTX3 promoter and the first enhancer, whereas the second PTX3 enhancer was not bound.

Among the regulatory regions analysed, the TFBS region and the first enhancer displayed the highest affinity for NF-kB.

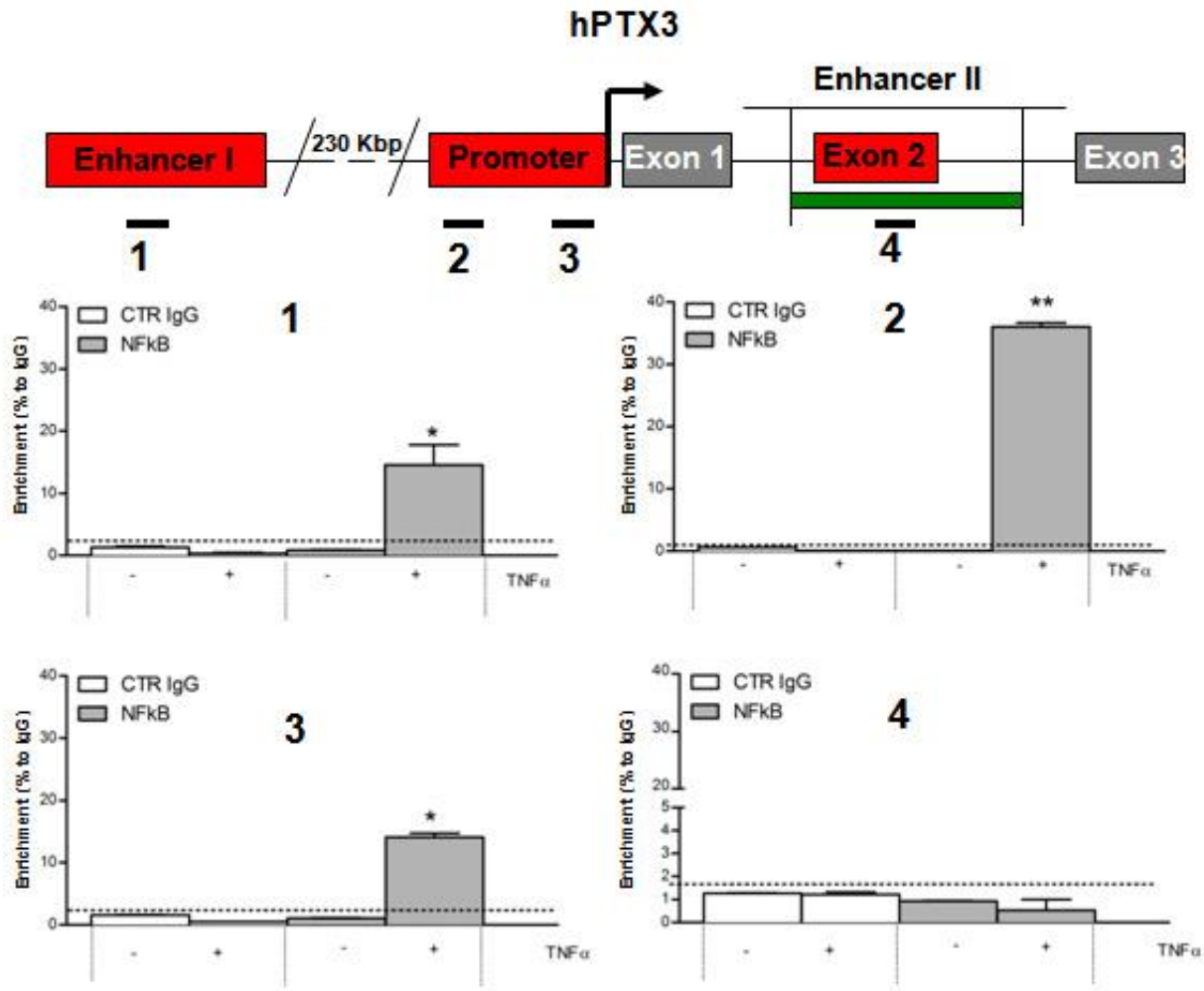


Figure 19: NF-kB binds PTX3 regulatory elements in the 8387 cell line.

ChIP assay in the 8387 cell line in basal condition and 4h after stimulation with TNFα. We analyzed PTX3 enhancer 1 (panel 1), promoter regions (panel 2 and 3), and enhancer 2 at the end of the CpG island (panel 4) using antibodies anti-NF-kB (p65). Results are expressed as percentage of enrichment to IgG and as a mean ± SEM (N = 3 experiments) of values obtained according to the formula 100 × 2^ (input Ct − sample Ct) and then normalized on IgG. *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$; Student's *t*-test. Each region analyzed with RT-qPCR is 150 bp long.

To characterize the mechanisms involved in the regulation of PTX3 expression, we performed a Chip assay for RNA Polymerase II (Pol II) and for the major Polycomb group subunities (SUZ12 and EZH2), in 8387 cells both in basal condition and after 4h of treatment with TNFα.

We analyzed the putative binding sites for the enzymes, using as negative control a region of the PTX3 intron 1. The results show that the promoter was active in basal condition and upon stimulation. An increased binding of RNA Pol II was observed, in particular in the proximity of the TSS (panel 3 figure 20).

The first enhancer did not bind Pol II, whereas bound SUZ12 and EZH2 in basal conditions. After inflammation, it lost the binding of the Polycomb complex and gained the binding of the RNA Pol II, thus getting active. The same results were obtained for the second enhancer, that bound the RNA Pol II only after inflammatory stimulus (panels 4 and 5 in the figure 20).

In one of the two regions considered (Panel 5, Figure 20), the RNA Pol II bound the second enhancer also in basal condition, but it was maintained paused by the Polycomb group. The stimulation with TNFα induced the loss of the Polycomb subunits and a strong increase of Pol II activity.

Collectively, these results indicate that both enhancer regions are inactive in basal conditions and became active after stimulation with TNFα.

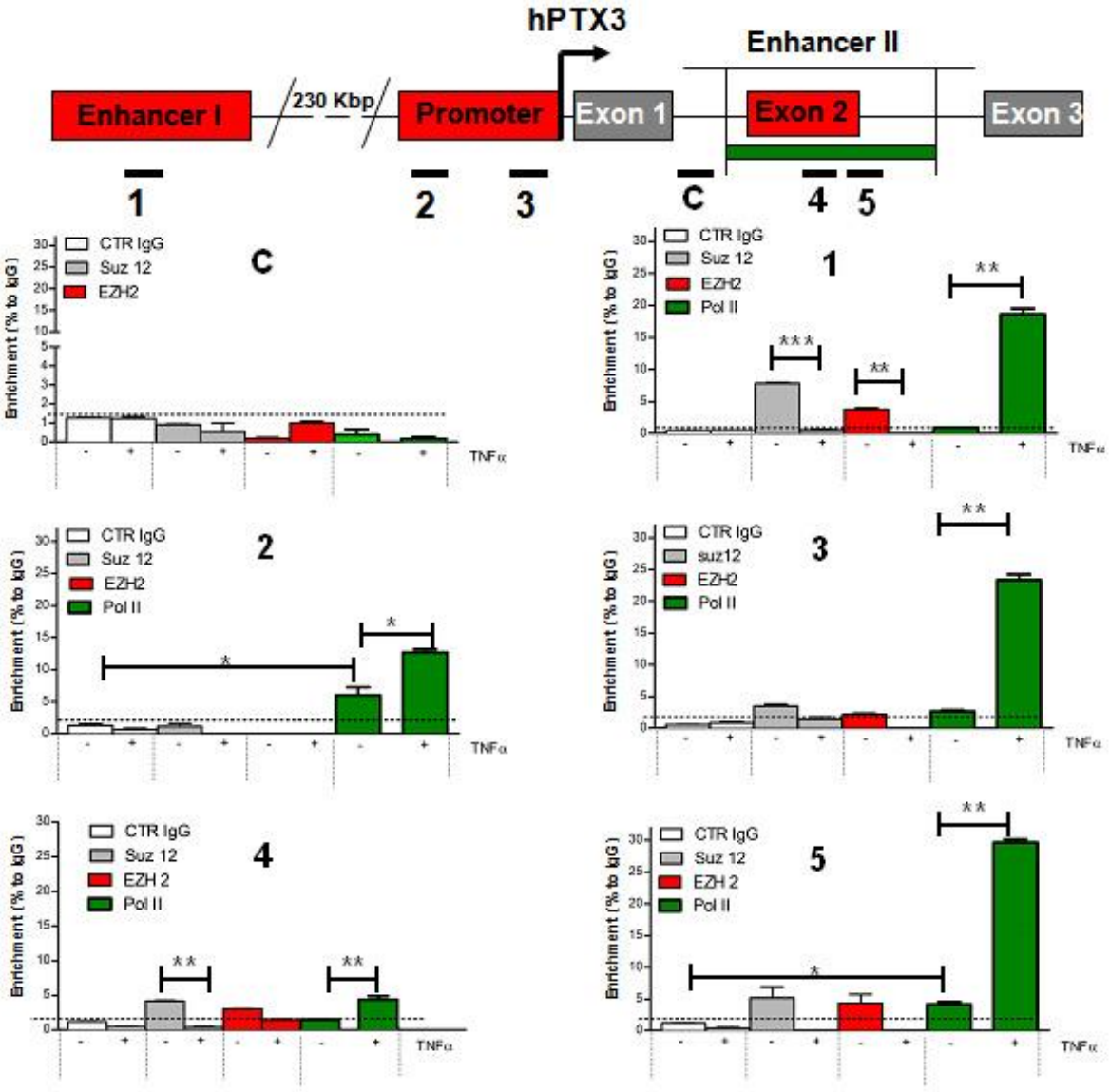


Figure 20: The Polycomb group regulates RNA Polymerase II activity in PTX3 regulatory regions.

ChIP assay in the 8387 cell line in basal condition and 4h after stimulation with TNFα in PTX3 intron 1 control region (panel C), enhancer 1 (panel 1), promoter regions (panel 2 and 3), enhancer 2 at the end of the CpG island (panel 4 and 5) using antibodies anti-Suz12, anti-EZH2, anti –RNA Pol II. Results are expressed as percentage of enrichment to IgG and as a mean ± SEM (N = 3 experiments) of values obtained according to the formula 100 × 2^ (input Ct − sample Ct) and then normalized on IgG. *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$; Student's *t*-test. Each region analyzed with RT-qPCR is 150 bp long.

2. PTX3 is directly and indirectly modulated by different miRNAs during inflammatory conditions.

The microRNAs (miRNAs) are small RNA non coding that silence several and multiple genes involved in different pathway, including in inflammation and immune response.

In order to verify the potential role of miRNAs in the regulation of PTX3 gene and the modulation of its network, we first confirmed that the miR 9, miR-29a/b/c and miR-181a/b/c/d, identified by bioinformatics softwares (Targetscan), actually targeted PTX3 3'UTR, performing a luciferase assay (figure 21 panels A-C). To address this point, we transfected 8387 cells with the mature miRNA and analysed PTX3 protein production 8h and 24h after stimulation with TNFα (figure 21 panel D), whereas mRNA expression was detected 4h and 8h after stimulation (figure 21 panel E). The results showed that miR-9, miR-29 family and miR-181 family significantly reduced PTX3 protein production both 8 hours and 24 hours upon stimulation with TNFα. Furthermore, PTX3 mRNA level was also reduced upon transfection with miR9, miR-29 family and miR-181a and miR-181c, after inflammatory stimulus. Thus suggest that these miRNAs were able to degrade their target.

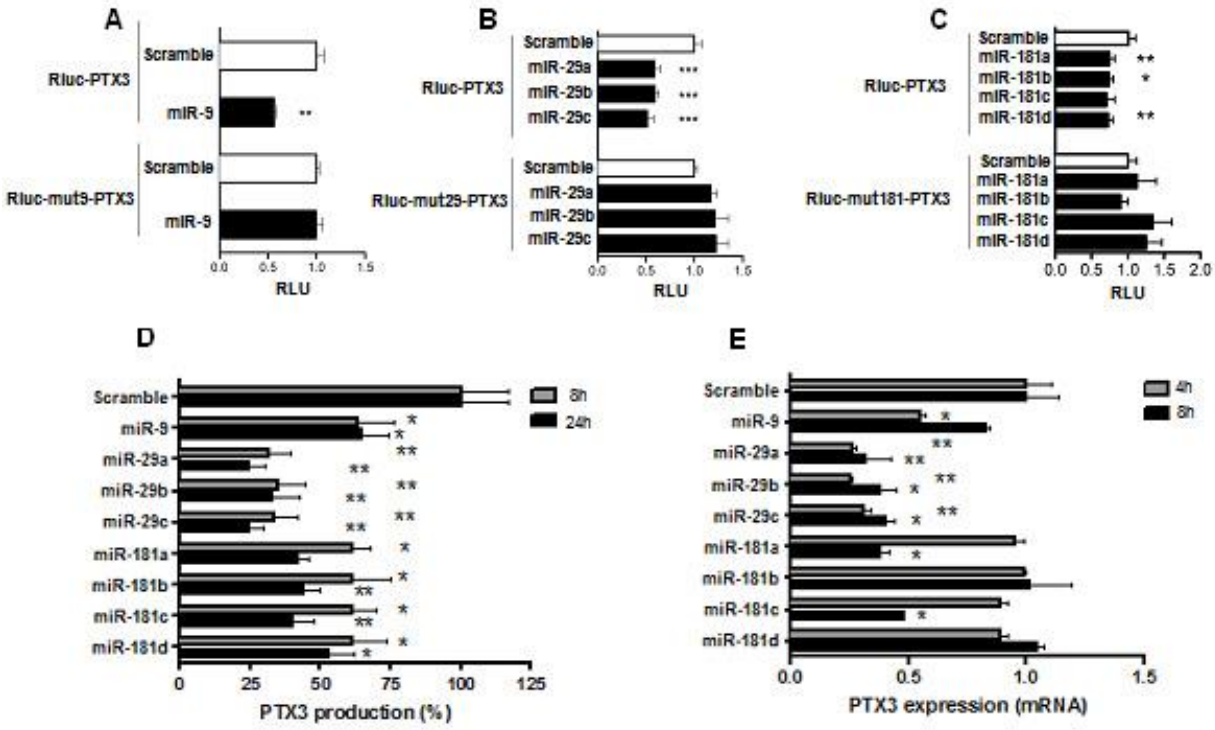


Figure 21: ***miR-9, miR-29 and miR-181 regulate PTX3***

A-B-C) Luciferase reporter assay in the HEK293T cells co-transfected with the indicated luciferase constructs (Rluc-vectors coding the PTX3 3'-UTR sequence, mutated in the seed region of each miR) together with miR-9, miR-29abc, miR-181abcd mimics or the scramble control (white column). Results are expressed as mean ± SEM (N = 3 experiments) of the ratio between *renilla* luciferase and *firefly* control luciferase activities (RLU) adjusted to 1. *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$; Dunnett's *post-hoc* test following a one-way ANOVA. D) TNFα-induced PTX3 production in 8387 cells 8 and 24 hours upon transfection with miR-9, miR-29c and miR-181c mimics or the scramble control. The data are shown as fold change and are obtained dividing by the scrumble and multiplying to 100. TNFα-induced (E) PTX3 expression at 4 and 8 h by 8387 cells transfected with miR-9, miR-29c and miR-181c mimics or the scramble control. The data are shown as . Results are shown as mean ± SEM (N = 3-6 experiments). *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$; Student's t-test.

To assess whether the regulation mediated by miR-9, miR-29 family and miR-18a family could also be an indirect mechanism, we cloned the PTX3 promoter into a specific luciferase vector to analyze its activity after 24h of stimulation with TNFα in 293T cell line, which does not express PTX3. All the microRNA analysed, except miR-9, determined a reduction of the promoter

activity (figure 22 panel A). Since the stimulation with TNFα induced the binding of NF-kB to the PTX3 promoter (figure 19), we transfected 293T cells with a NF-kB reporter vector, in order to check whether the miRNAs considered had an effect on the transcription factor activity (figure 9 panel B). Results showed that miR-181 family and miR-29b reduced NF-kB activity.

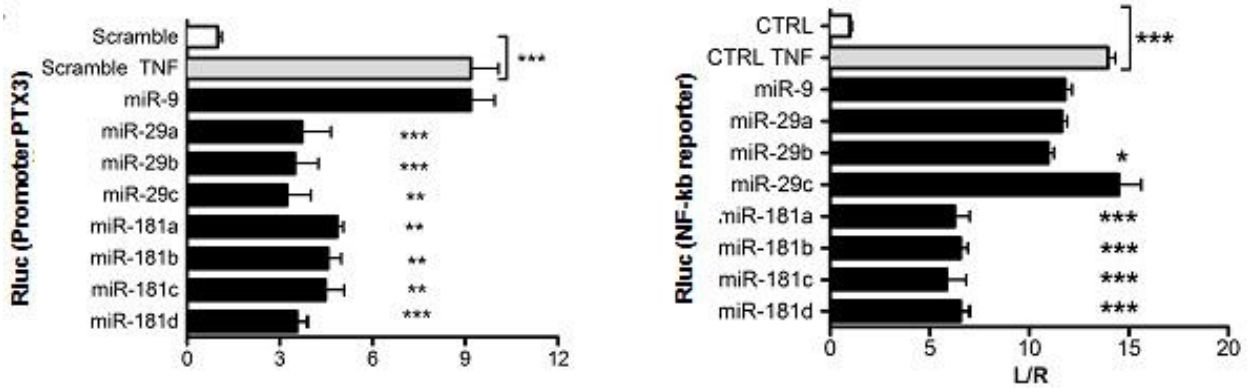


Figure 22. miRs regulate PTX3 promoter

A) Luciferase reporter assay in the HEK293T cell line co-transfected with the PGV-B2 vector expressing the *PTX3* promoter sequence fused to the firefly luciferase Coding sequence and the pRL-TK vector expressing *renilla* luciferase as normalizer and with the specified miR mimics (black bars) or the scramble control (white bars) (A). The experiment was conducted in basal condition and upon stimulation with 20 ng/mL TNFα . Results were expressed as mean ± SEM (N = 3 experiments) of the ratio between *firefly* luciferase and *renilla* control luciferase activities (L/R). *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$; Dunnett's *post-hoc* test following a one-way ANOVA. (B) Luciferase reporter assay in the HEK293T cell line co-transfected with the NF-kB reporter vector expressing NF-kB regulatory sequence fused to the firefly luciferase CDS and the pRL-TK vector expressing renilla luciferase as normalizer and with the specified miR mimics (black bars) or the scramble control (white bars).

Several studies showed that the innate immune response and also the inflammatory response are regulated by miR-9, miR-146a, miR-155 and miR132. More in details, these inflammatory miRNAs might modulate the inflammatory transcription factors NF-kB. We checked whether miR-146a, miR-155 and miR132, regulate PTX3 expression. The experiments demonstrated that these miRNAs downregulated PTX3 promoter activity (figure 23 panel A), acting on NF-kB

pathway (figure 23 panel B), and thus affecting the PTX3 promoter activity (figure 23 panel C and D). miR-146a, miR-155 and miR-132 did not target PTX3 3'UTR (figure 23 panel E)

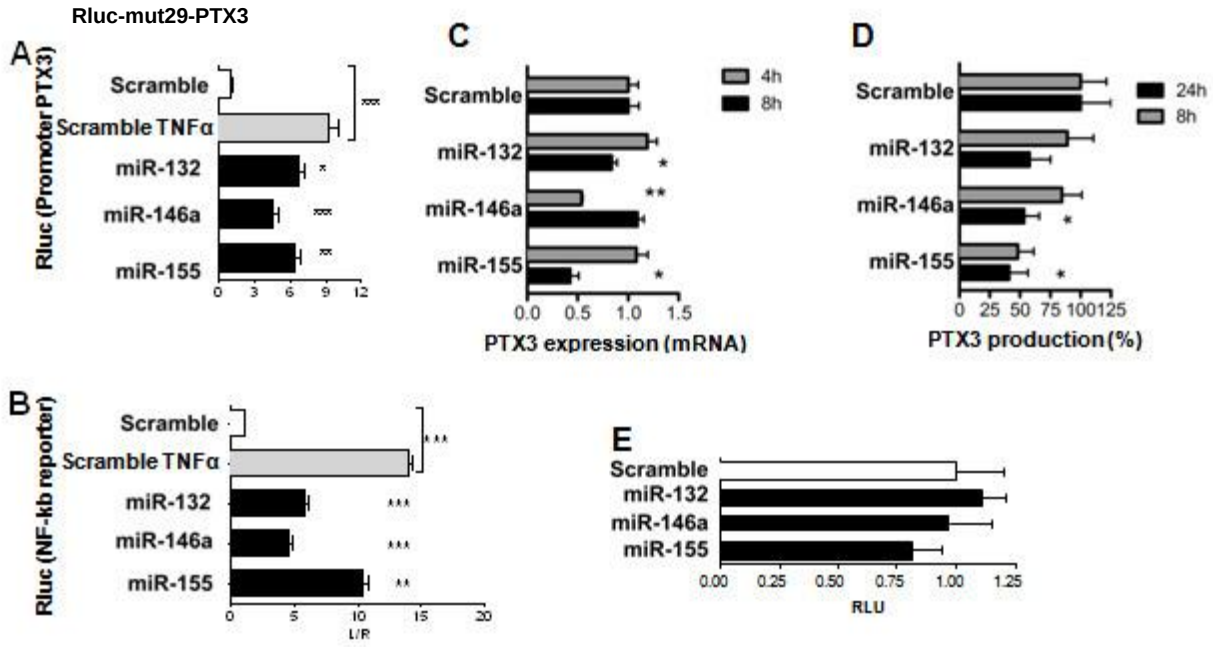


Figure 23. Inflammatory miRNAs indirectly regulate PTX3 expression.

A-B)Luciferase reporter assay in HEK293T cell line co-transfected with PGV-B2 vector expressing the *PTX3* promoter sequence (A) and with NF-kB reporter vector expressing NF-kB regulatory sequence (B) fused to the firefly luciferase CDS and pRL-TK vector expressing *renilla* luciferase as normalizer and with the specified miR mimics (black bars) or the scramble control (white and grey bars). The experiment was conducted upon stimulation with 20 ng/mL TNFα

C-D)TNFα-induced PTX3 mRNA expression , at 4h and 8h after treatment with TNFα (C), and production (D) at 8h and 24h after treatment with by 8387 cell lines transfected with miR-132 , miR-146a , miR-155 mimics or the scramble control. Results are shown as mean ± SEM (N = 3-6 experiments). *: $p \leq 0.05$, **: $p \leq 0.01$; Student's *t*-test.

E) Luciferase reporter assay in the HEK293T cells co-transfected with the indicated luciferase constructs (Rluc-vectors coding the PTX3 3'-UTR sequence) together with miR-132, miR-146a and miR-155 mimics or the scramble control (white column). Results are expressed as mean ± SEM (N = 3 experiments) of the ratio between *renilla* luciferase and *firefly* control luciferase activities (RLU) adjusted to 1. *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$; Dunnett's *post-hoc* test following a one-way ANOVA.

In order to improve the characterization of PTX3 regulatory network, we took advantage of the IPA Ingenuity software (http://www.ingenuity.com/products/ipa), focusing on inflammatory pathways. IPA tools allow to analyze published interaction data, uncovering multi-level direct and causal relationships, thus including regulators that are not directly connected to targets.

We analyzed PTX3 pathway, focusing on the interactions of PTX3 with several inflammatory cytokines, including IL-1β and TNFα, and the bacterial component LPS (Figure 24).

The analysis also included the putative PTX3 regulatory miRNAs calculated with mathematics algorithms and validated in our study. We obtained a complex interaction network in which PTX3 was regulated by inflammatory cytokines, but could also regulate those cytokines. Indeed, the graph showed that PTX3 could indirectly regulate its own expression. PTX3, LPS and the cytokines analyzed, could activate TLR4 and TLR9 pathway, modulating several genes downstream of the TLR. Finally, the microRNAs that directly targeted PTX3 were predicted to regulate transcription factors, LPS and some inflammatory cytokines, as well.

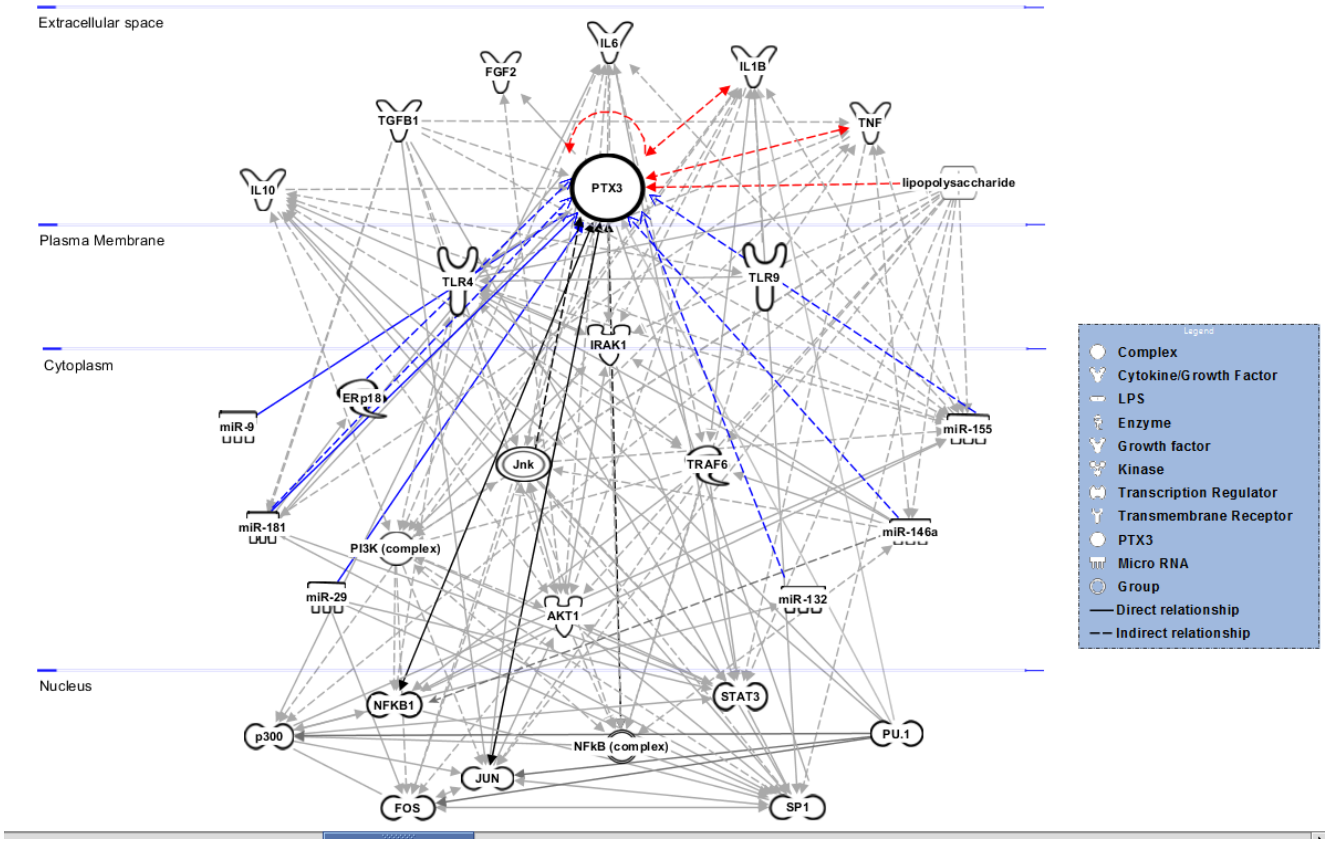


Figure 24: PTX3 interaction pathway.

Interaction network of PTX3 protein in inflammatory pathway extrapolated by IPA Ingenuity software. The protein interaction data are extrapolated to publish articles, whereas the regulation of the miRNAs are calculated by also mathematics algorithms.

Finally, to validate the data obtained and to better define the mechanism of action of microRNAs, we performed a RNA ImmunoPrecipitation assays (RIP) on human resting or LPS-stimulated macrophages, at different time point. We used an antibodies that recognises the Ago2 protein and thus the RISC complex which binds the mature miR. The results show that PTX3 expression increased 4 hours upon stimulation and started decreasing after 6 hours. At the same time, the entrance of both PTX3 and the presence of the miRNAs to the RISC was observed, 6 hours upon stimulation (figure 25). Interestingly, in basal condition the miRNAs had no effect on PTX3 expression.

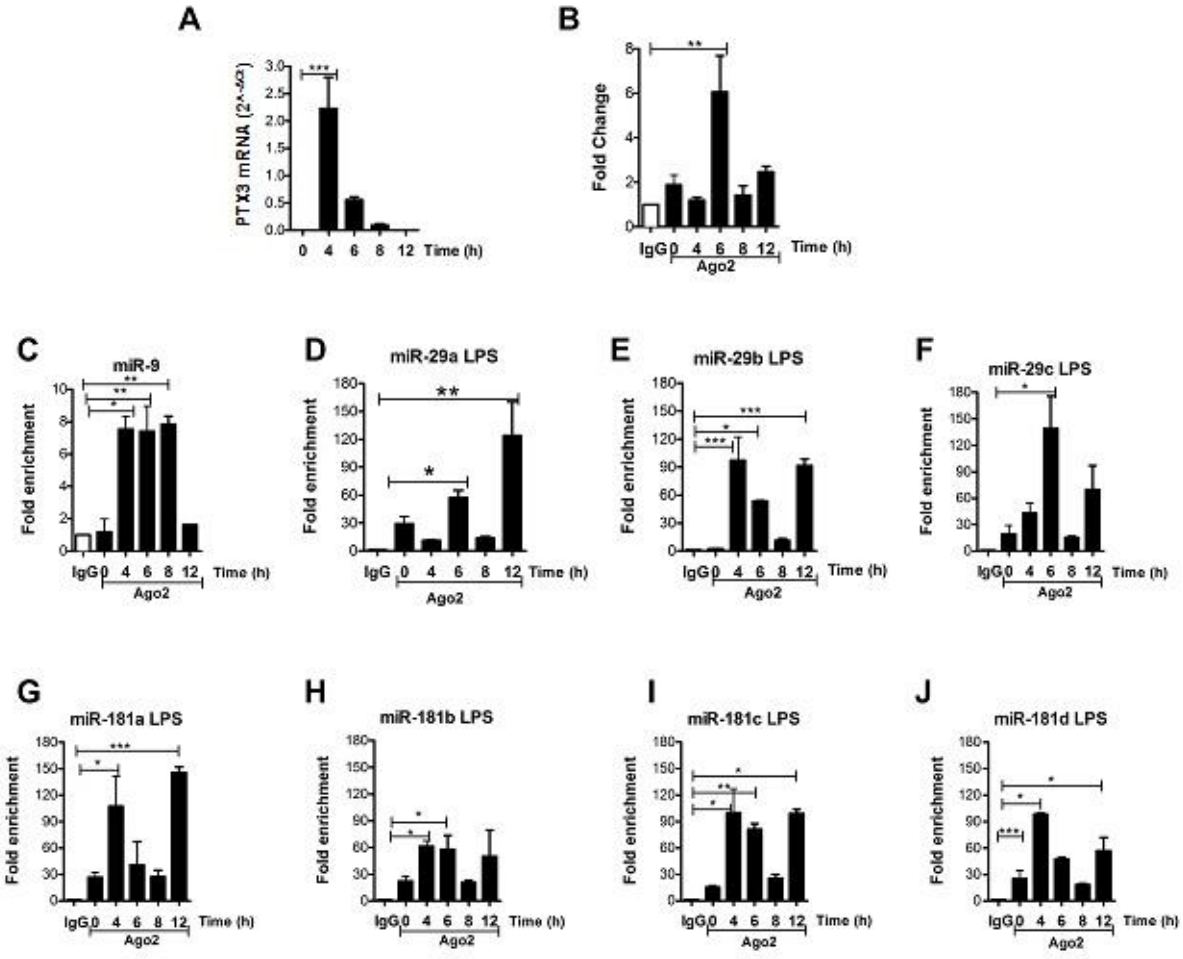


Figure 25. PTX3 is directly regulated by several miRNAs in macrophages after LPS stimulation.

Resting macrophages and after stimulation with 100 ng/mL LPS were subjected to RIP assay by using anti-Ago-2 or IgG control Abs at different time points.

A) Kinetic analysis by RT-qPCR of PTX3 mRNA expression in human macrophage after stimulation with LPS. Result are expressed as $2^{\Lambda-\Lambda Ct}$ and as mean ± SEM (N = 3 experiments). *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$; Student's *t*-test.

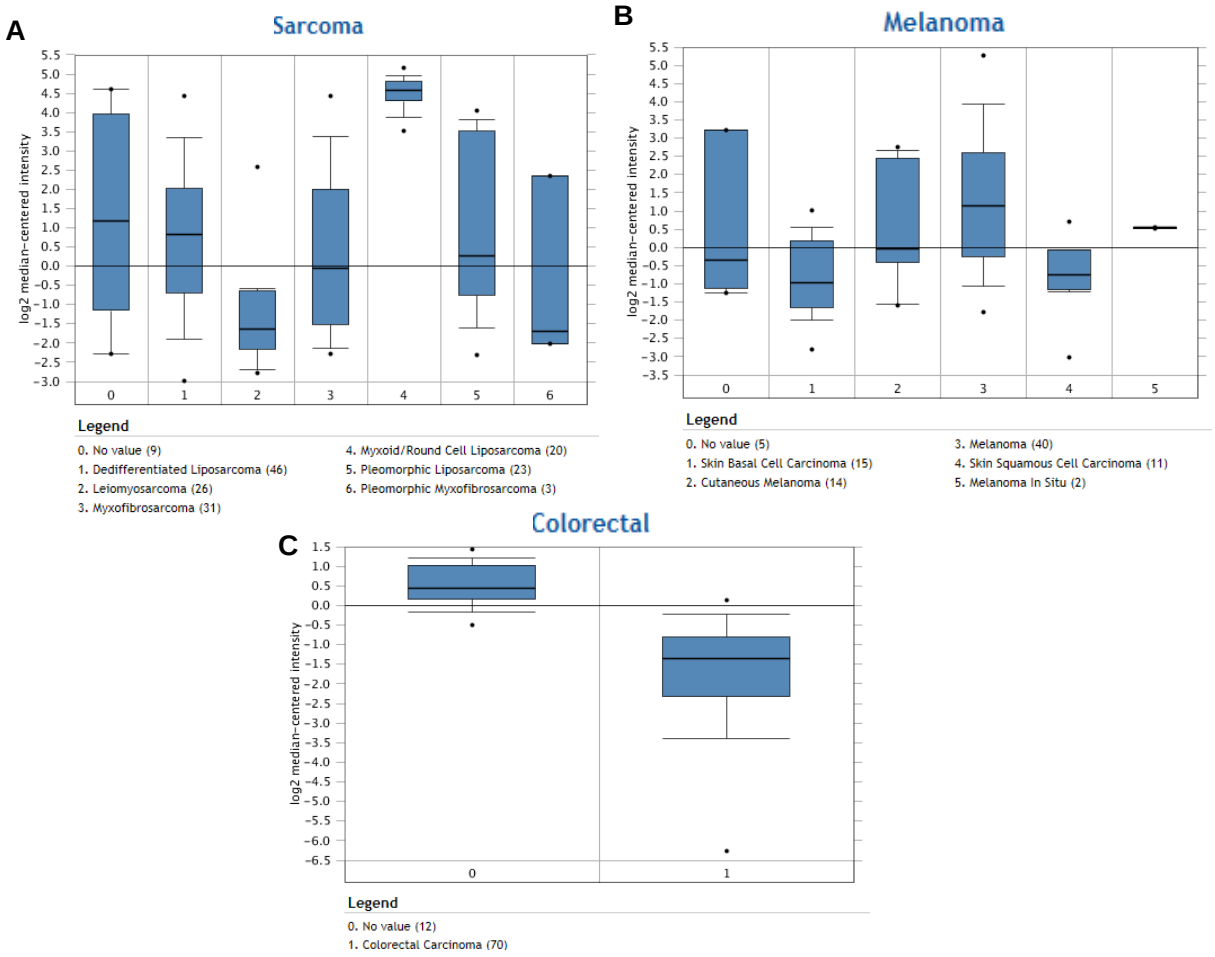
B-J) Levels of PTX3 (B) and miRNAs (C-J) detected in the RISC complex were determined by RT-qPCR in IP. Results are expressed as fold enrichment relative to the corresponding IgG control sample (mean ± SEM, N = 3 donors). *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$; Student's *t*-test.

3.PTX3 regulatory mechanisms in cancer

3.1 PTX3 expression in cancer

Preclinical data obtained in PTX3 KO mice studying epithelial and mesenchymal cancerogenesis demonstrated that PTX3 played a key role in controlling tumor incidence.

In order to assess whether the results obtained in the mouse could be relevant in humans we evaluated PTX3 mRNA expression level in human tumor tissues. We used the Oncomine browser and checked PTX3 mRNA expression in both mesenchymal- and epithelial-derived tumors. As Figure 26 shows, PTX3 mRNA expression level in normal tissues and in different sarcoma tumors (figure 26 panel A) [184], melanoma (figure 26 panel B) and epithelial tumors, like skin cancer (figure 26 panel B) [185] and colorectal cancer (figure 26 panel C) [186]. The data indicate that PTX3 expression is variable, depending on the type of tissue and also on the type of tumor. For example, sarcoma tumors analyzed displayed similar levels of PTX3 mRNA compared to the healthy tissue, except leiomyosarcoma, in which the level of PTX3 transcript was lower (figure 26, panel A column 2). Healthy epithelial tissues generally displayed low levels of PTX3 mRNA (figures 26 panel B and C, column 0), compared to the other tissue types analyzed. Moreover, the PTX3 transcript level was reduced in both skin and colon cancer compared to the healthy counterpart.



Figures adapted from Oncomine**.**

Figure 26: PTX3 expression in tumors .

A) Barretina Sarcoma Dataset: PTX3 mRNA expression was measured in 149 soft tissue sarcomas of 6 different subtypes and 9 normal adipose tissue (number 0: no value) specimens.

B) Riker Melanoma Dataset: PTX3 mRNA expression was measured in 82 tissue of patients with 5 different subtypes melanoma and 5 normal epithelial tissue (number 0: no value)

C) Hong Colorectal Dataset: PTX3 mRNA expression was measured 70 colorectal carcinoma and 12 normal colon samples.

3.2 PTX3 is silenced in colorectal cancer cells through DNA methylation

To confirm the data from Oncomine, we analysed PTX3 expression in several colorectal cancer (CRC) cell lines in basal condition and upon treatment with inflammatory stimuli. To verify the potential role of DNA methylation as main actor involved in PTX3 silencing, we treated CRC

cells with the DNA methylation inhibitory agent 5'AZA- 2' deoxycytidine (5'AZA-dC). With this treatment, we obtained a rescue of PTX3 mRNA expression and protein production (Figure 27), whereas the co-treatment with DNA methylation inhibitor and inflammatory cytokines determined higher PTX3 level compared to the healthy colon. Conversely, the co-treatment with the inhibitor of the histone deacetylases Trichostatine A (TSA) and 5'AZA-dC had the same effects as the inhibitor of methylation alone. We also checked PTX3 mRNA expression levels in healthy epithelial colon cell line (HCoEpic) in inflammatory conditions with or without 5'aza-dC treatment (figure 27).

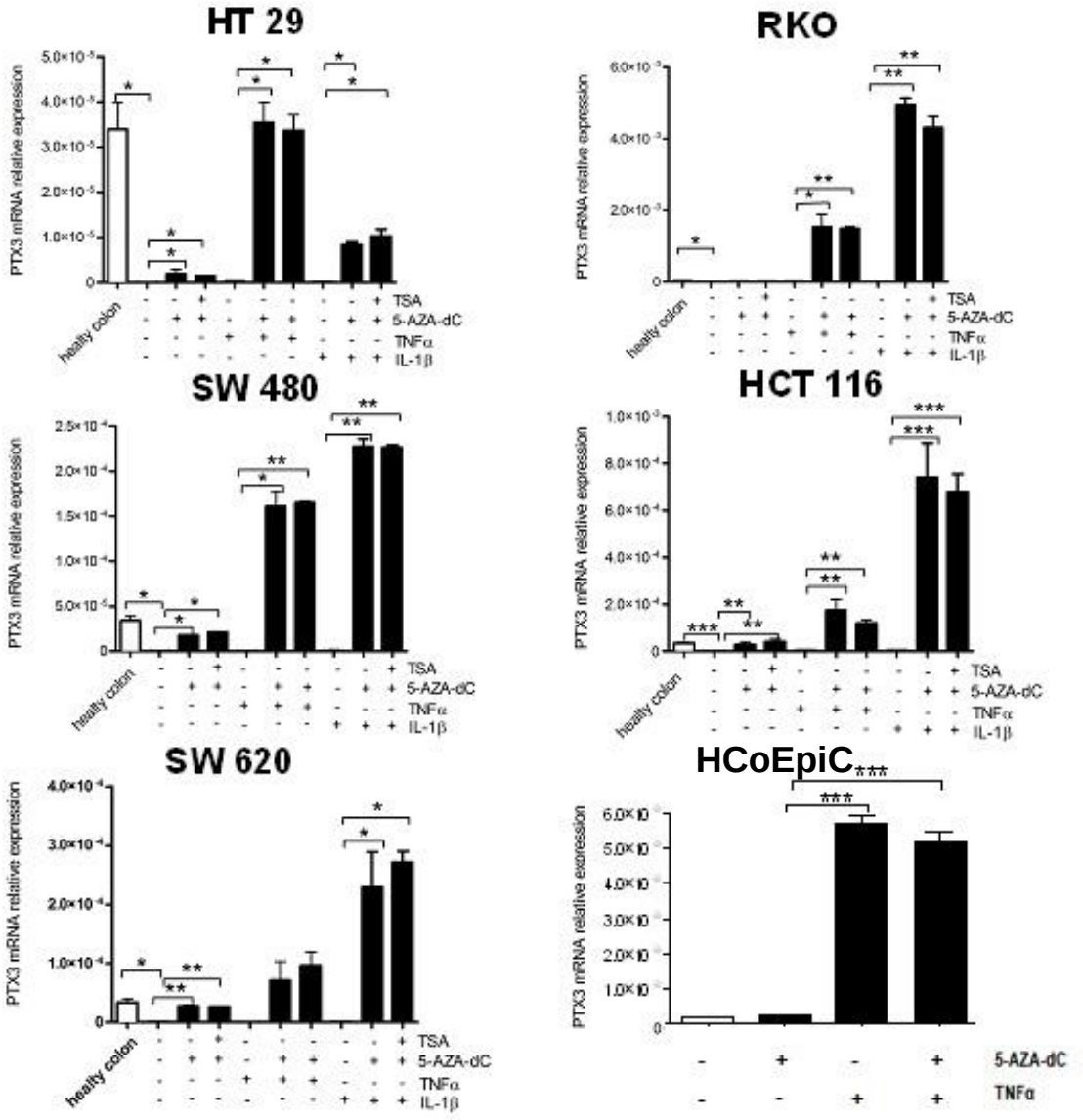


Figure adapted from Bonavita E, Gentile S., **Rubino M**, et al. Accepted for publication in Cell 2015

Figure 27: PTX3 expression is restored byco-treatment with 5'AZA-dC and TNFα in CRC cell lines.

PTX3 mRNA expression by SW480, HT29, RKO, HCT116, SW620 and normal colon epithelial cell line (HcoEpic). CRC cell lines were treated or not with 5'AZA-dC alone and with 5'AZA-dC and TSA in the presence of TNFα or IL-1β. HcoEpic were treated with 5'AZA-dC alone and with TNFα. Result are expressed as $2^{\wedge-\Delta Ct}$ and as mean ± SEM (N = 3 experiments). *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$; Student's *t*-test.

We also analyzed the effect of the inhibitor of DNA methylation on PTX3 production in the CRC cell lines that were more responsive to 5'AZA-dC treatment (figure 28). The inhibitory agent together with inflammatory cytokines restored the levels of PTX3 protein, whereas 5'AZA-dC alone had a slight effect on PTX3 rescue.

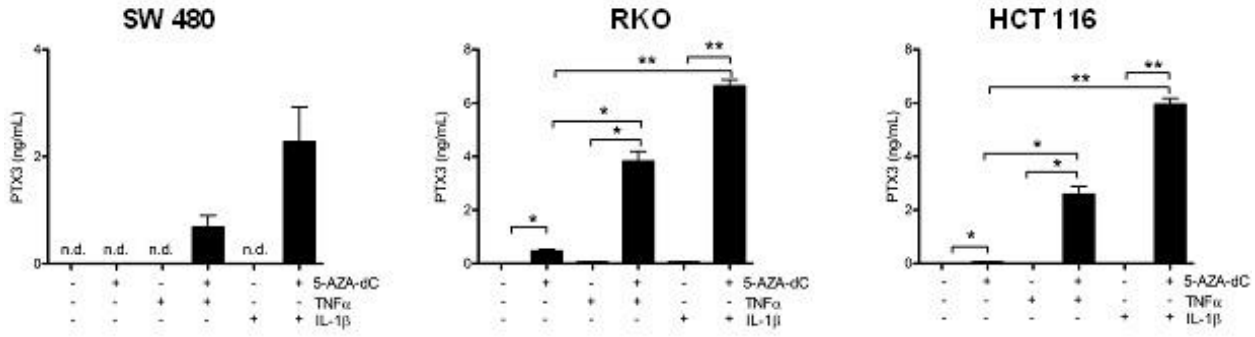


Figure adapted from Bonavita E, Gentile S., **Rubino M**, et al. Accepted for publication in Cell 2015

Figure 28: PTX3 protein production is restored in CRC cell lines after cotreatment with 5'AZA-dC and TNFα.

PTX3 mRNA protein production by SW480, RKO and HCT116 cell lines treated or not with 5-AZA-dC in the presence of TNFα 20 ng/mL or IL-1β 20 ng/mL. Result are expressed ang/ml and as mean ± SEM (N = 3 experiments). *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$; Student's *t*-test.

We analysed *PTX3* methylation in 4 CRC cell lines of different tumor stages (I-IV). As shown in Figure 29 (panel A) PTX3 promoter region and the second enhancer were hypermethylated, whereas the first enhancer was hypometylated. The treatment with the methylation inhibitor 5-AZA-dC significantly reduced the methylation of PTX3 regulatory regions.

To verify if PTX3 methylation detected in CRC cells was dependent on the tumor or on the type of tissue, we checked DNA methylation levels of a human epithelial colon cell line (HCoEpiC) (figure 29 panel B). The data showed that Human colonic ephitelial cells were hypomethylated, with a methylation levels lower than 5% in promoter region and corresponding to the CpG island (second enhancer), whereas the first enhancer was completely unmethylated.

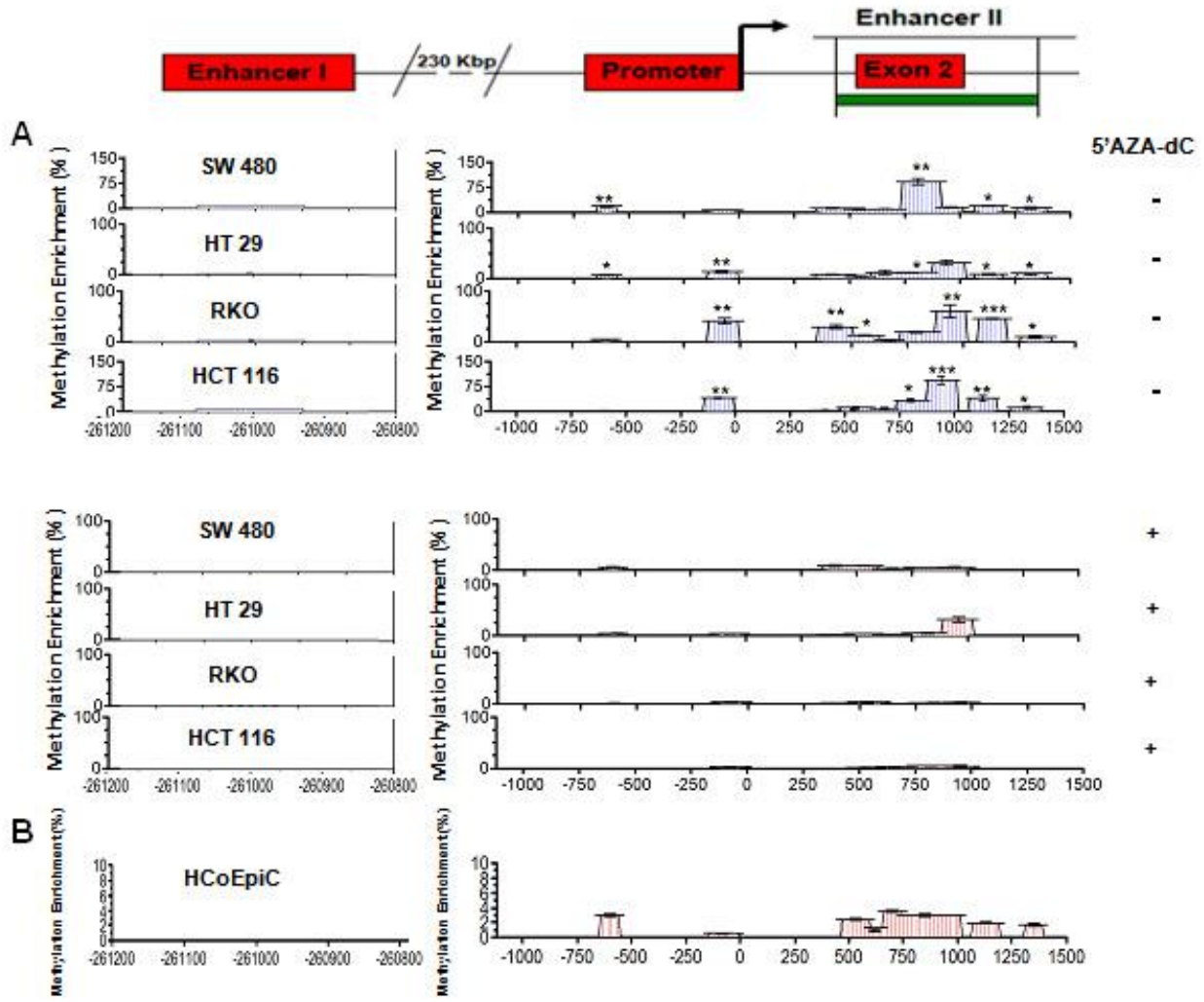


Figure adapted from Bonavita E, Gentile S., **Rubino M**, et al. Accepted for publication in Cell 2015

Figure 29: PTX3 is hypermethylated in CRC cell lines.

A) Analysis by MIRA of the percentage of methylation enrichment of PTX3 regulatory regions in CRC cell lines with and without treatment with 5'AZA-dC.

B) Analysis by MIRA of the percentage of methylation enrichment of PTX3 regulatory regions in HcoEpiC cell line.

Results are expressed as percentage of enrichment relatively to input DNA normalized on a positive control and represented as mean ± SEM. *: p<0.05, **: p<0.01, ***: p<0.001, unpaired student's t test.

Finally, to define the possible involvment of microRNA in PTX3 regulation in CRC, we used the bioinformatics browser mimiRNA (http://mimirna.centenary.org.au/mep/formulaire.html) to analyze the expressions of miRNA of our interest in healthy colon tissue and in colon malignant tumor. The analysis showed that there were not change in miRNA expression in healthy and colon tumor tissues (data not shown), indicating that miRNAs are not involved in the regulations of PTX3 in this tumor.

3.3 PTX3 regulatory regions became active in CRC cells upon co-treatment with 5'aza-dC and TNFα.

Given that we observed a rescue of PTX3 expression upon treatment with 5'AZA-dC, we wondered whether it could be due to an effect on PTX3 regulatory regions. Thus, we performed a ChIP assay in the two CRC cell lines which were more responsive to treatment with the methylation inhibitor (RKO and HCT116, figures 27 and 28), in several stimulation conditions.
PTX3 enhancers were inactive in CRC cells in basal conditions and upon treatment with inflammatory stimuli, whereas an increase in the levels of active markers and a reduction in the levels of repression markers was observed upon treatment with 5'AZA-dC (figure 30 panel A and B). The most significant activation was achieved after co-stimulation with the methylation inhibitor and TNFα, in both cell lines.

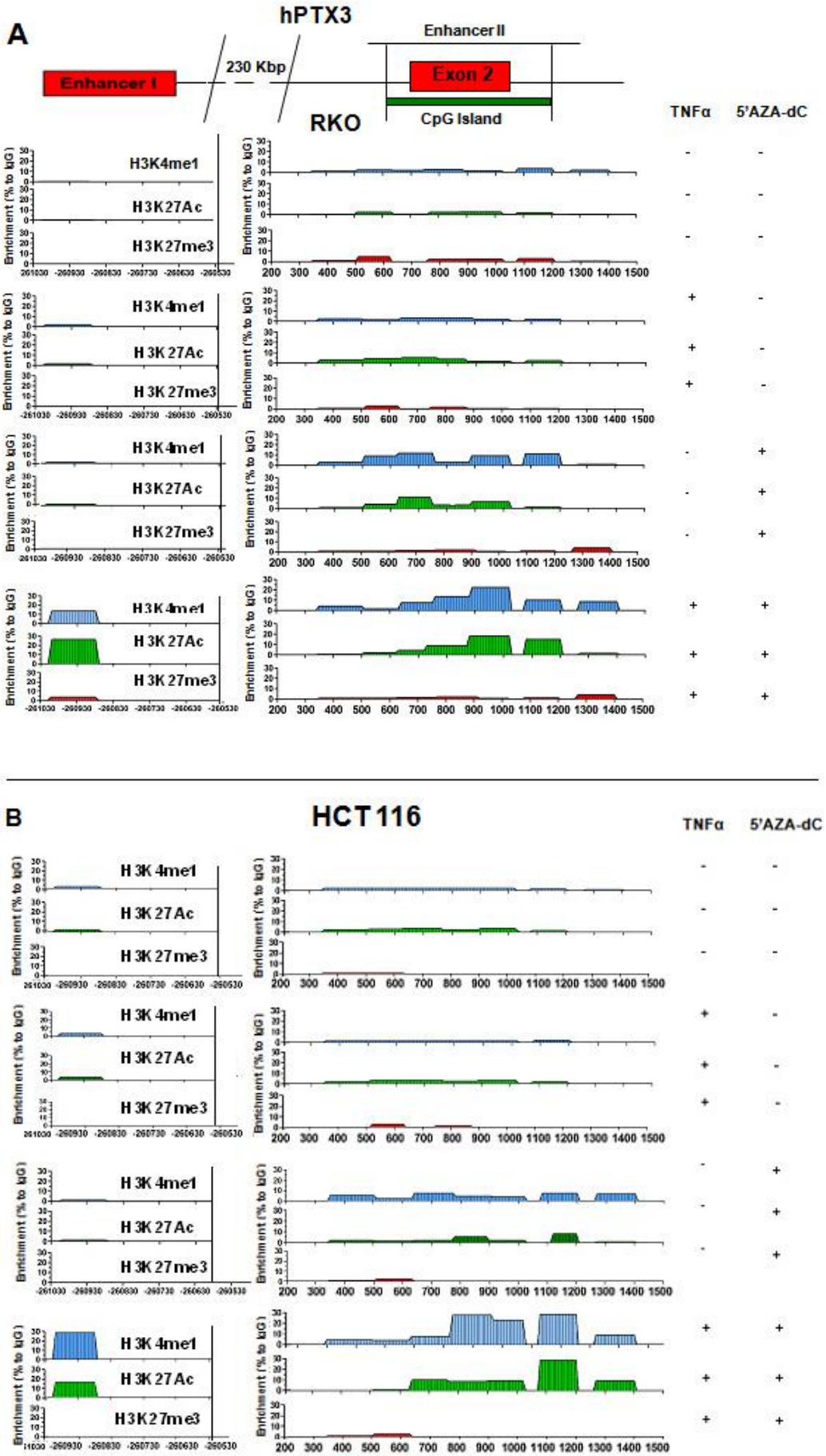

A
hPTX3
Enhancer II
Enhancer I
230 Kbp
Exon 2
CpG Island
RKO
TNFα
5'AZA-dC
H3K4me1
H3K27Ac
H3K27me3
Enrichment (% to IgG)
B
HCT116
TNFα
5'AZA-dC
H3K4me1
H3K27Ac
H3K27me3
Enrichment (% to IgG)

Figure 30: PTX3 enhancer are inactive in CRC cell lines but become active after cotreatment with 5'AZA-dC and TNFα

ChIP assay in the RKO cell line (A) and HCT116 cell line (B) in basal condition, 4h after stimulation with TNFα, 72h after treatment with 5'AZA-dC, and after cotreatment with 5'AZA-dC and TNFα, using antibodies recognizing H3K4me1, H3K27Ac and H3K27me3 histone modifications. Results are expressed as percentage of enrichment to IgG and as a mean ± SEM (N = 3 experiments) of values obtained according to the formula 100 × 2^ (input Ct − sample Ct) and then normalized on IgG. *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$; Student's *t*-test. Each region analyzed with RT-qPCR is 150 bp long

Then, to gain insight into the activity of PTX3 regulatory regions in colon cells and to confirm the data obtained in CRC cells, we performed a ChIP assay in human epithelial healthy colon cells (HCoEpiC), using Abs which characterize enhancer activity and also Abs specific for RNA Pol II and for SUZ12. We decided to focus on SUZ12 other than HEZ2 because of the higher affinity of the antibody observed in the previous experiments (figure 20).

In basal conditions enhancers were poised (figure 18) because of the presence of H3K27me3 and SUZ12, whereas, after treatment with TNFα, they became active, thus losing Polycomb and gaining the binding of H3K4me1, H3K27Ac and RNA Polymerase II.

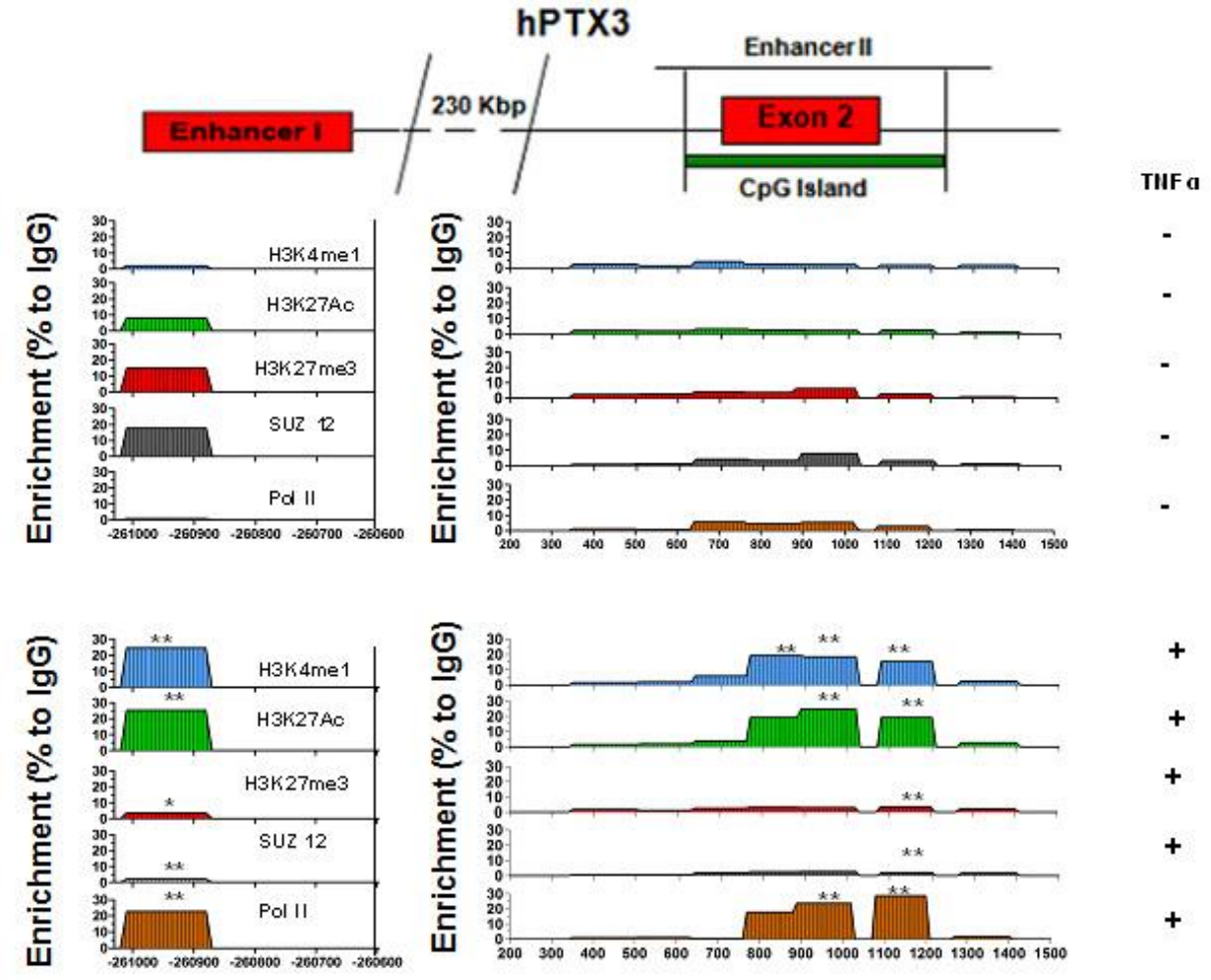


Figure 31: PTX3 enhancers are poised in HCoEpiC cell lines and became active after treatment with TNFα.

ChIP assay in the HCoEpiC cell line in basal condition and 4h after stimulation with TNFα, using antibodies recognizing H3K4me1, H3K27Ac and H3K27me3 histone modifications and for SUZ12 and RNA Pol II. Results are expressed as percentage of enrichment to IgG and as a mean ± SEM (N = 3 experiments) of values obtained according to the formula 100 × 2^ (input Ct − sample Ct) and then normalized on IgG. *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$; Student's *t*-test. Each region analyzed with RT-qPCR is 150 bp long

In line with these results, PTX3 promoter activity showed a similar response to the different stimuli in CRC cells: we observed activation upon treatment with 5'AZA-dC and even a stronger activation in presence of inflammatory cytokines (Figure 32 panel A and B).

In the PTX3 promoter of healthy colon cells the levels of H3K4me3, H3K9AC and RNA Pol II in basal conditions (Figure 32 panel C) significatively increased, after treatment with TNFα, thus favouring the binding of Pol II.

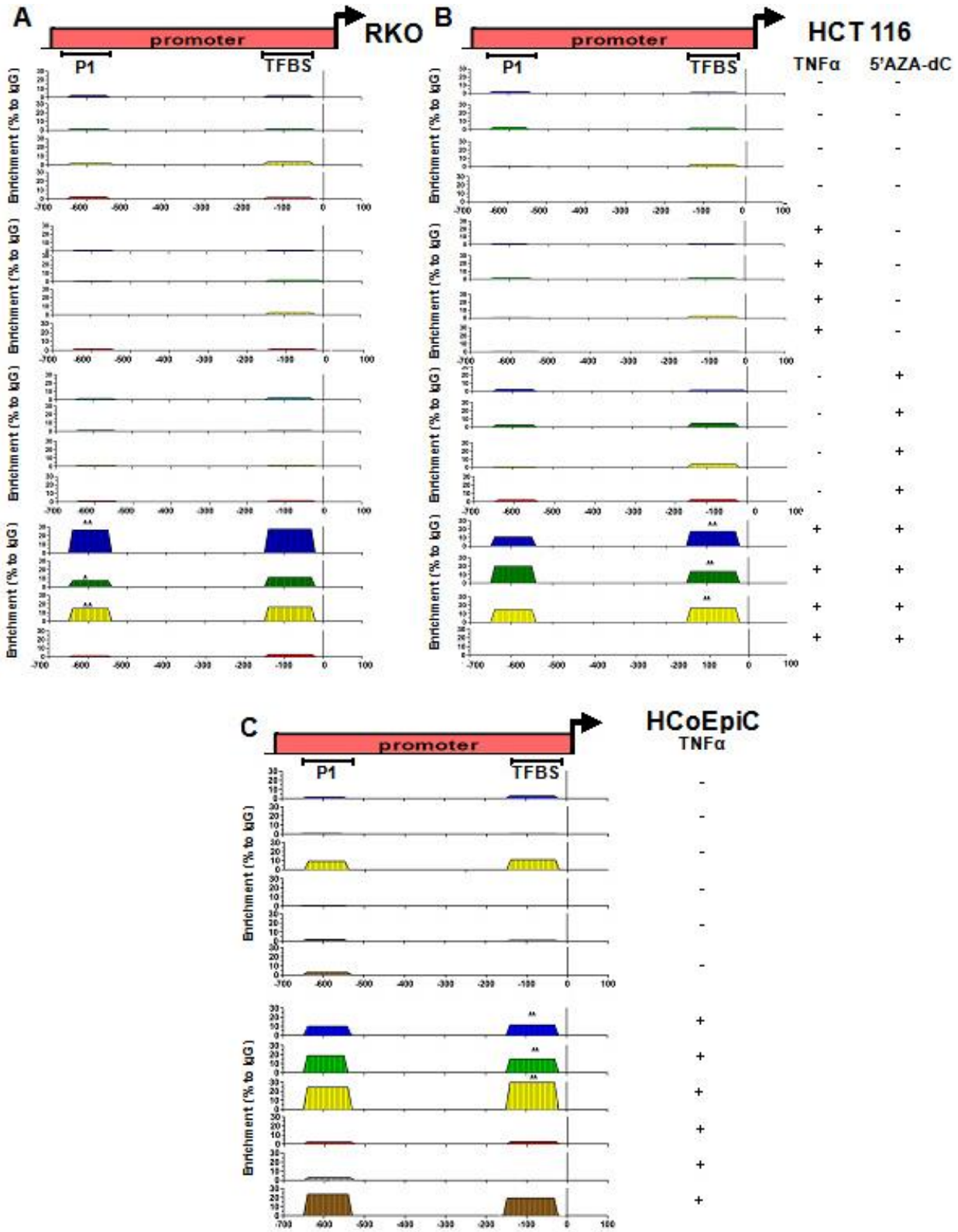


Figure 32: PTX3 promoter is no active in CRC cell lines until co-treatment with 5'AZA-dC and TNFα.

A-B) ChIP assay in the RKO cell line (A) and HCT116 cell line (B) in basal condition, 4h after stimulation with TNFα, 72h after treatment with 5'AZA-dC, and after cotreatment with 5'AZA-dC and TNFα, using antibodies recognizing H3K4me3, H3K27Ac, H3K9Ac and H3K27me3 histone modifications.

C) ChIP assay in the HCoEpiC cell line in basal condition and 4h after stimulation with TNFα, using antibodies recognizing H3K4me3, H3K27Ac, H3K9Ac and H3K27me3 histone modifications and for SUZ12 and RNA Pol II. Results are expressed as percentage of enrichment to IgG and as a mean ± SEM (N = 3 experiments) of values obtained according to the formula 100 × 2^ (input Ct − sample Ct) and then normalized on IgG. *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$; Student's *t*-test. Each region analyzed with RT-qPCR is 150 bp long

Finally, we analyzed the binding of inflammatory transcription factors NF-kB and the AP-1 subunits c-Fos and c-Jun regulate PTX3 expression. The data demonstrated that demethylation ~~is~~ was responsible for the binding of NF-kb and AP-1 to the PTX3 promoter in inflammatory conditions whereas the first enhancer only bound NF-kb, as we previously demonstrated in the 8387 cell line (figure 19).

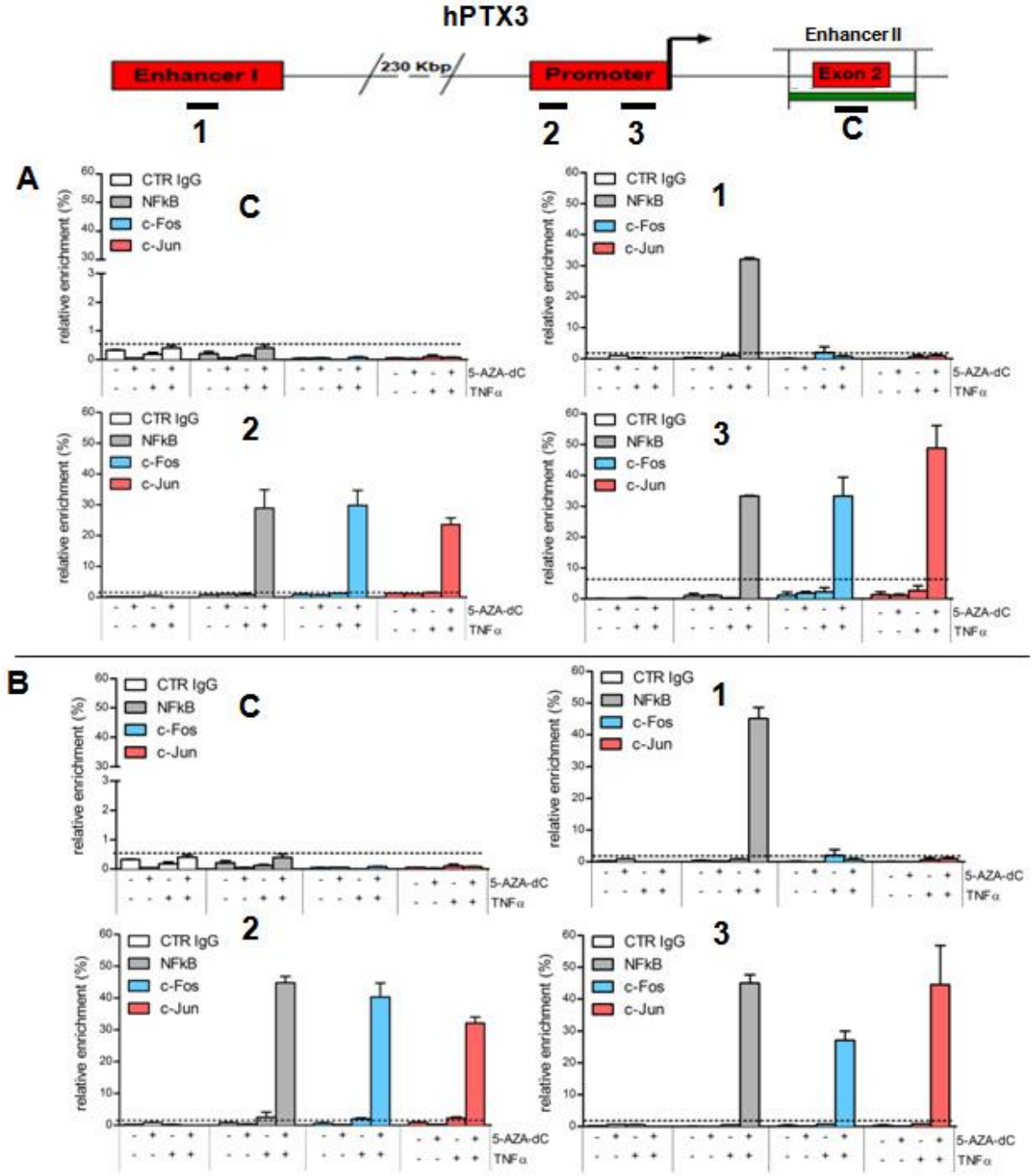


Figure adapted from Bonavita E, Gentile S., **Rubino M**, et al. Accepted for publication in Cell 2015

Figure 33: inflammatory transcription factors bind the PTX3 promoter and the first enhancer in CRC cell lines after co-treatment with 5'AZA.dC and TNFα.

A-B) ChIP assay in the RKO cell line (A) and in HCT116 cell line (B) in basal condition and 4h after stimulation with TNFα, 72h after treatment with 5'AZA-dC, and after cotreatment with 5'AZA-dC and TNFα . We analyzed PTX3 enhancer 1 (panel 1), promoter regions (panel 2 and 3), and enhancer 2 at the end of the CpG island (panel 4) using antibodies anti-NF-kB (p65). Results are expressed as percentage of enrichment to IgG and as a mean ± SEM (N = 3 experiments) of values obtained according to the formula 100 × 2^ (input Ct − sample Ct) and then

normalized on IgG. *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$; Student's *t*-test. Each region analyzed with RT-qPCR is 150 bp long.

3.4 PTX3 is hypermetilated in several human tumors.

In order to confirm in vivo the results obtained from CRC cell lines, we extended the methylation analysis to CRC samples derived from patients with colon cancer at different stages (stage 1 to 4), as well as in adenoma (Figure 34). For each tumor stage we analyzed also the corresponding healthy tissue. The data demonstrated that PTX3 regulatory regions were signifatively methylated in all tumour stages compared to their healthy counterpart. Moreover, the methylation started to adenoma.

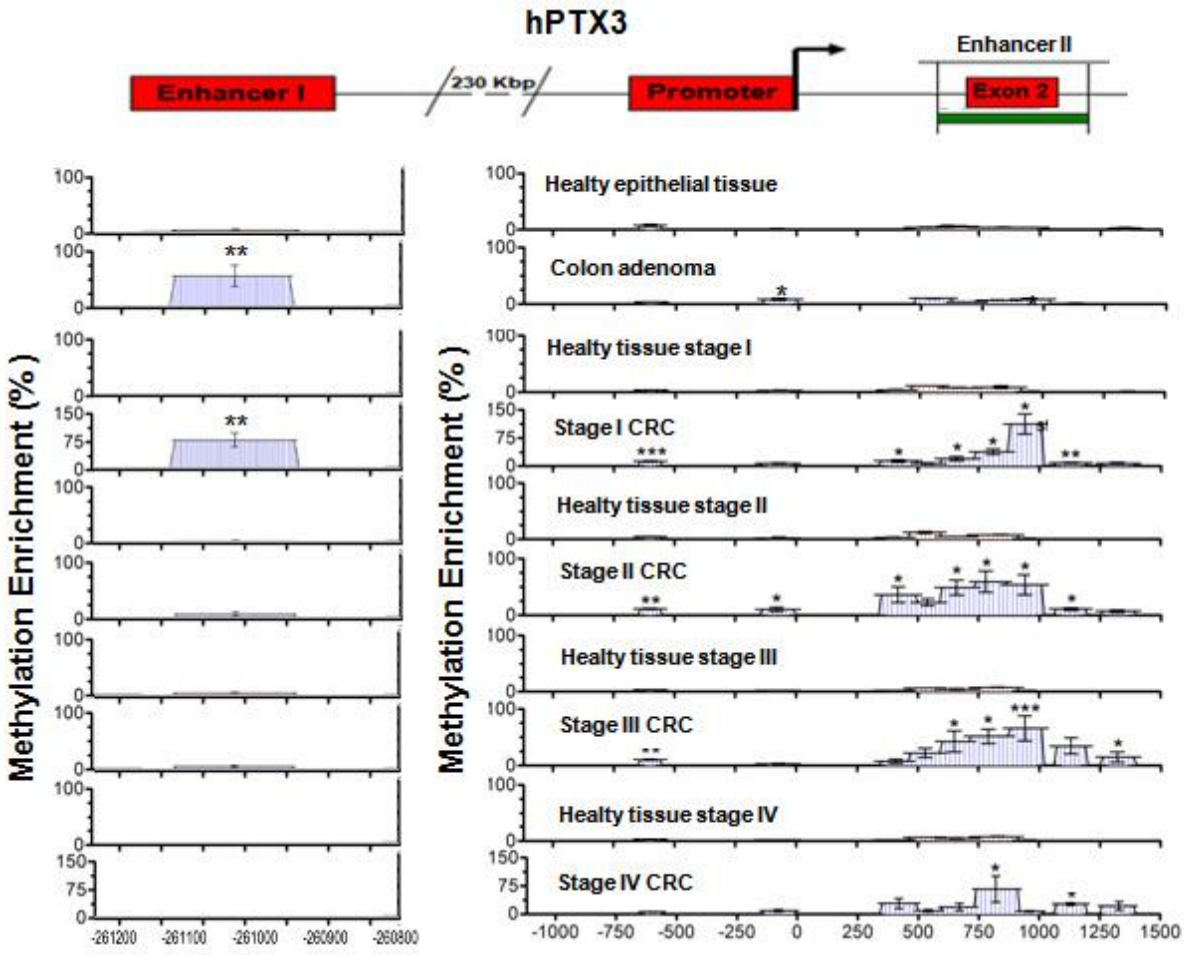


Figure adapted from Bonavita E, Gentile S., **Rubino M**, et al. Accepted for publication in Cell 2015

Figure 34: PTX3 regulatory regions are methylated in CRC patients compared with their healthy counterpart.

Analysis by MIRA of the percentage of methylation enrichment of the promoter region and of the *PTX3* CpG island in healthy colon epithelium, 5 high grade adenomas, 10 CRC and 2 healthy counterparts.

Results are expressed as percentage of enrichment relatively to input DNA normalized on a positive control and represented as mean ± SEM. *: p<0.05, **: p<0.01, ***: p<0.001, unpaired student's t test.

We further checked PTX3 methylation levels in mesenchimal, including angiosarcoma, synovial sarcoma, leiomysarcoma, solitary fibrous tumour (SFT), chordoma, gastrointestinal stromal tumor (GIST), and desmoids tumors and in epithelial tumors, like squamous cell carcinoma (Figure 22). We also analyzed PTX3 methylation levels in both mesenchymal and epithelial healty tissue. As you can see in Figure 35, PTX3 regulatory regions were hypermetilated in different human tumors.

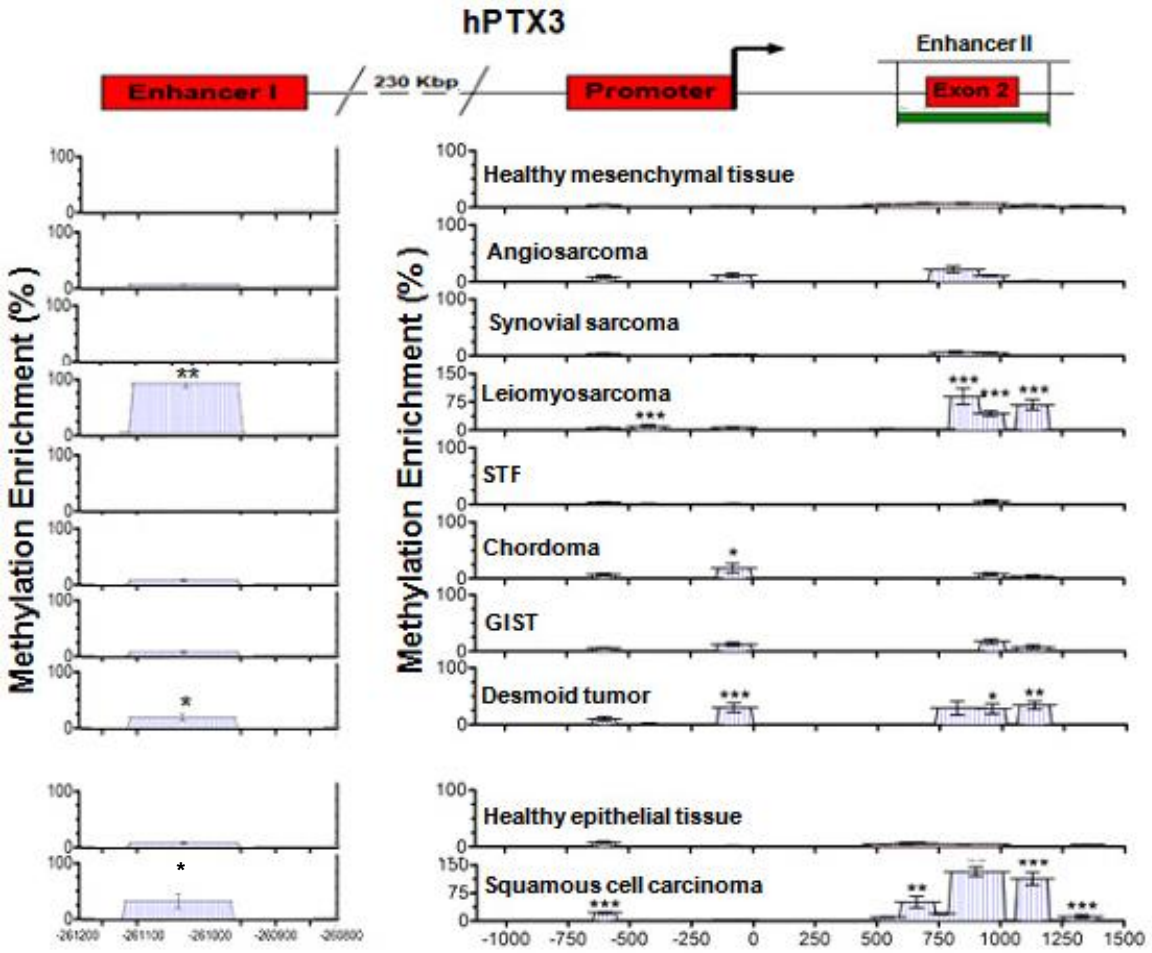


Figure adapted from Bonavita E, Gentile S., **Rubino M**, et al. Accepted for publication in Cell 2015

Figure 35: PTX3 regulatory regions are methylated in several human tumors.

Analysis by MIRA of the percentage of methylation enrichment of the promoter region and of the *PTX3* CpG island in human mesenchymal and epithelial tumors: Skin healthy epithelial tissue from healthy counterparts of skin squamous carcinoma (n=5), healthy mesenchymal tissue from leiomyosarcoma (n=1), angiosarcoma (n=3), chordoma (n=3) and desmoid tumor (n=2) patients and 3-8 samples.

Results are expressed as percentage of enrichment relatively to input DNA normalized on a positive control and represented as mean ± SEM. *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$, unpaired student's t test.

3.5 PTX3 methylation levels inversely correlate with PTX3 production in tumor samples.

To further correlate PTX3 methylation in tumors with the lack of PTX3 expression in vivo, we performed immunohistochemistry assay in CRC samples and in their corresponding healthy counterpart. As shown in Figure 36, in healthy tissue in association with epithelial cells, we detected PTX3, which was hypomethylated in that tissue (figure 34). In contrast in CRC samples the protein was product by inflammatory cells and stroma cells but was not associated to epithelial tumor cells, which were found to be hypermetilated at levels of PTX3 enhancers and promoter.

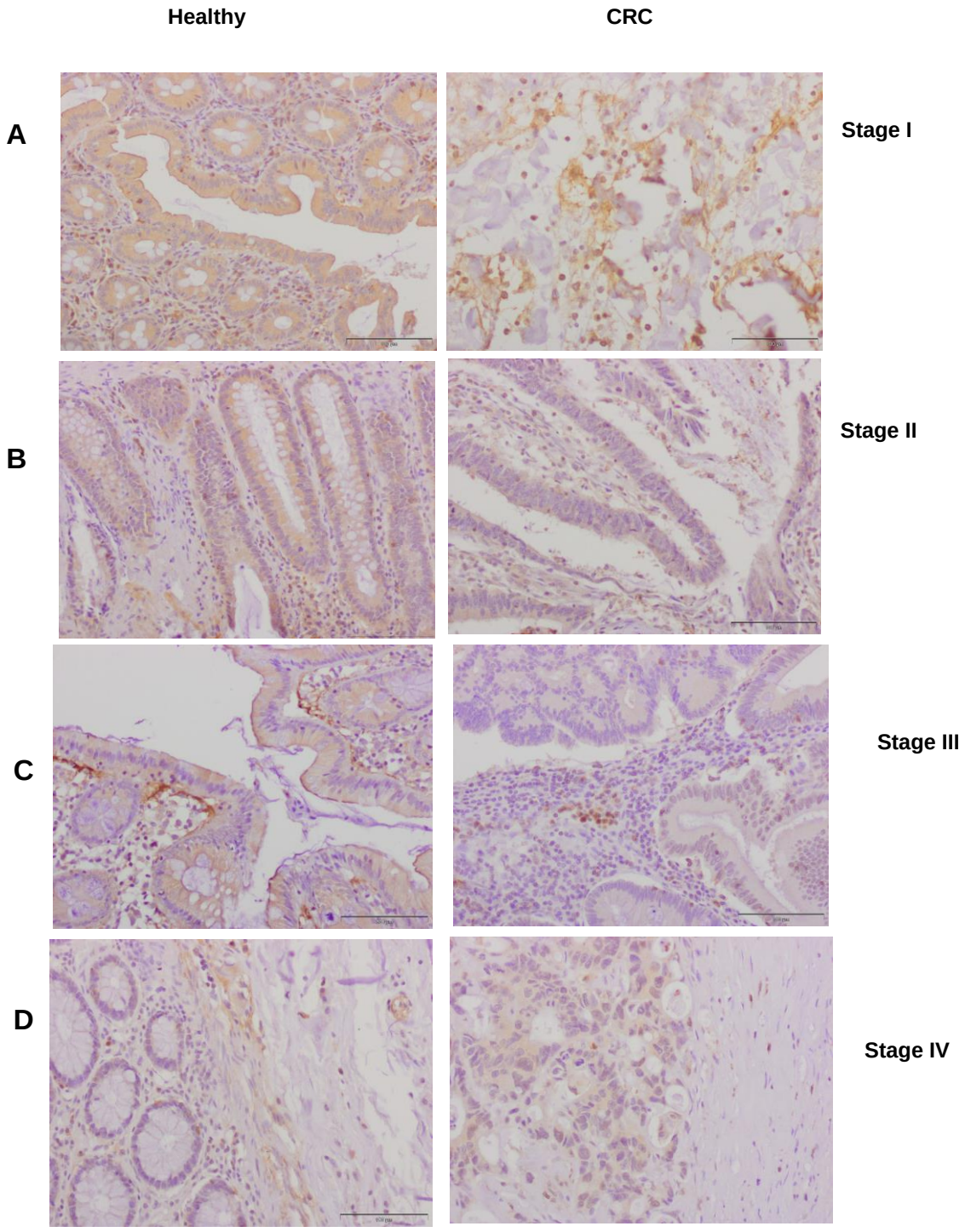


Figure 36: PTX3 is express in healthy epithelial colon tissue but it's not express in CRC cells.

A-D) PTX 3 expression assessed by immunohistochemistry staining in colorectal cancer tumor tissues and adjacent nontumor tissues.

Discussion:

- PTX3 expression is regulated by two enhancers during acute inflammation.

In this study we investigated the mechanisms involved in the regulation of the expression of the long pentraxin PTX3 protein in physiological conditions, in inflammation and in cancer. We found that PTX3 trascription was regulated by 2 enhancers, highly conserved among species. The first one maps at 230 kb upstream of the TSS of the PTX3 promoter region and the second enhancer localized with the second exon of the gene.

The long pentraxin PTX3 is highly regulates in different cell types, in particular in mononuclear phagocytes. PTX3 is poorly expressed in basal conditions, rapidly increases in the acute phase of inflammation responses and then its levels decrease, to basal levels in hours. PTX3 mRNA expression correlates with the activity of 2 different enhancers identified in this study. Indeed when PTX3 is highly express, after inflammatory stimulation, the enhancers are both active, whereas in basal condition they are poised and the promoter is the only active regulatory region. ChIP assay for transcription factors demonstrated that, after inflammatory stimuli acting through the TLR4 ad MyD88 pathways, both the first enhancer and the promoter binds the inflammatory transcription factor NF-kB, which regulate PTX3 gene expression. Given that differentiated cells have a unique repertoire of enhancers [187-191] characterized by specific transcription factors (TFs), we hypothesize that the first enhancer is activated and thus can regulate PTX3 expression, only during inflammatory conditions. In line with this hypothesizes, only in inflammatory conditions the first enhancer acquires the binding for RNA polymerase II. Thus we infer that this long-range enhancer might form a chromatin loop on the PTX3 promoter region, increasing its binding to Pol II and thus the gene expression.

The second enhancer, as well as the PTX3 promoter, binds the RNA polymerase II both in basal condition and upon stimulation with TNFα, thus suggesting a functional role of the Pol II in the enhancer activity, or in the elongation of the PTX3 mRNA transcript.

In basal conditions, PTX3 enhancers displayed both H3K27me3 repressor histone modifications and SUZ12 and EZH12 Polycomb Repressor Complex 2 (PRC2) subunit. This is in line with the results of different studies, which demonstrated that low-active genes can bind both RNA Pol II and PRC. PRC2 is a negative chromatin remodeler complex which is able to close the chromatin structure through H3K27me3, generated by EZH1/2 methyltransferasis. This complex can also reduce the activity of the RNA Pol II. [192,193]. Thus, we hypothesized that in basal condition, PRC2 could be responsible of silencing of PTX3 enhancers. Upon stimulation with TNFα, the regulatory regions showed an increased Pol II activity, an enhanced gene expression and the lost of PCR2 subunits.

Collectively, these data suggest that the high expression of PTX3 in inflammatory conditions depends on activation of enhancers, whereas the gene is poorly active in basal condition through PRC2-dependant inactivation of the enhancers.

- miRNAs modulate PTX3 activity in inflammatory conditions through direct and indirect mechanisms.

MicroRNAs are regulators of numerous genes and pathways, can modulate the immune response, and play a crucial role in the regulation of inflammatory and autoimmune diseases and cancer.
We confirmed with luciferase assays that PTX3 3‘UTR was targeted by 8 different microRNAs, as predicted by bioinformatics analysis. In particular, the most relevant seem to be mir-9 and miR-29 family, which reduced the luciferase activity by more than 40%, and the members of the miR-181 family which reduced luciferase activity by about 30%.
We also tested the effect of these miRNAs on PTX3 expression at different time points after treatment with TNFα. MiR-181 family and in particular miR-29 family had a stronger effect on PTX3 protein expression at 24 hours, whereas miR-9 had a comparable effect at both time points.
The analysis of PTX3 mRNA showed that some miRNAs could also reduce the messenger levels, maybe through the decay of the messenger or via indirect mechanisms, acting on the signalling pathways leading to PTX3 transcription.
MiR-29 family reduced PTX3 messenger and protein production more than the other miRNAs. Among miR-181 family, only miR-181a and miR-181c had an effect on PTX3 mRNA, suggesting that each miRNA can target different genes, as reported in literature [194].
The analysis of miRNA effects on PTX3 promoter through luciferase assay confirmed that both miR-29 and miR-181 families reduced the promoter activity, thus affecting the signalling pathway which induces PTX3 expression. In agreement, miR-181 family and miR-29b reduced the activity of NF-kB.
Collectively, these data indicate that the miRNA-dependent reduction of PTX3 mRNA is principally due to their role in the inhibition of transcription factors involved in PTX3 expression. Moreover, the study of the effects of inflammatory miRNA on PTX3 gene revealed that they regulated NF-kB, affecting both gene expression and production, in agreement with published

data [195]. This effect likely reflects at least in part the direct targeting by these miRNAs of molecules involved in signalling cascades culminating in the transcriptional activation of *PTX3*, including TRAF6 and IRAK1 for miR-146a [195] and MyD88 and TAB2 for miR-155 [196]

In line with these hypotheses, protein interactions analysis, performed with IPA ingenuity software, revealed that PTX3 is part of a complex network: PTX3 can indirectly modulate the cytokines and the transcriptions factors that in turn regulate its expression and also modulate the activity of several miRNAs. This kind of feedback-regulation was previously proposed by Anya Rudnicki [197] and was then extended in our study. The complex and strong regulation of PTX3 at different levels gives evidence of the great importance of this gene in the modulation of the inflammatory response, suggesting its potential role as hub gene.

The RNA ImmunoPrecipitation (RIP) of the RISC in LPS stimulated macrophages allowed us to demonstrate the actual occurrence of aggregation of PTX3 mRNA and miRNAs acting directly on the PTX3 gene with the RISC. Indeed, PTX3 mRNA expression started reducing 6 hours upon treatment with LPS, the time necessary for its aggregation with the RISC. The microRNA of our interest were also highly associated with the complex at that time point: miR-29c and miR-181c in particular. Indeed, these two miRNAs, that were present at high levels in the RISC complex, bound also PTX3 3'UTR with a major efficiency compared to the other members of their family.

Interestingly, the levels of all the miRNAs in the RISC decreased 8 hours after treatment with LPS, in line with PTX3 expression in the complex. Collectively, these data suggest that PTX3 is highly regulated by several miRNAs, including has-miR-224 detected by Anya Rudnicki and other ten miRNAs discovered in our study, that can act both in a direct or an indirect way, with a different specificity, mechanism and time of action.

-The methylation of the enhancers silence PTX3 gene in human cancer.

In vivo studies in our laboratories demonstrated that PTX3-deficiency increases susceptibility to cancer development and growth in MCA-induced fibrosarcoma and DMBA-TPA induced skin cancer models. Thus, in these models PTX3 has a protective role against tumor.

The analysis performed with Oncomine browser showed that PTX3 gene is expressed at low levels in several human cancer tissues compared to the healthy counterparts.

Collectively, these data suggest that PTX3 might be involved in the pathogenesis of human cancers and the lack of his expression in several tumors could be due to alterations of regulatory mechanisms.

In the last years, an increasing number of studies demonstrated that epigenetic mechanisms, together with genetic alterations, play a crucial role in the regulation of cancer development. Epigenetic alterations were proposed to be key regulators of the initiation of tumorigenesis [28, 198]. In particular, the cancer epigenome is characterized by global DNA hypomethylation and site-specific hypermethylations that affect regulatory elements of oncosuppressor genes.

Methylation assays on the PTX3 gene showed that CRC cells, which did not express PTX3, were hypermetylated, whereas healthy colon epithelial cells were hypomethylated and expressed PTX3. We analyzed histone modifications that characterize gene regulatory elements, in CRC cells after several treatments and stimulations. The data obtained in 2 different lines indicated that the PTX3 promoter and the first enhancer became active only after 5'aza-dC and TNFα co-treatment. Treatment with 5'aza-dC alone partially reverted PTX3 mRNA expression and protein production and induced only the activation of the PTX3 intragenic enhancer. This indicated that the second PTX3 enhancer, which in healthy cell lines bound RNA Pol II, both in basal and in inflammatory conditions, is sufficient to regulate PTX3 expression.

Indeed, recent studies demonstrated that RNA Polymerase II activity started on enhancers and after moved on target promoter. Some groups suggested that the components of the transcription

machinery and the preinitiation complex (PIC) were recruited to the enhancer. [199-201]. Only upon formation of the PIC on the enhancer, the complex can move along the promoter.

Taken together, these data suggest that PTX3 intragenic enhancer is the key regulator of PTX3 gene expression and it also controls the activity of both the promoter and long-range enhancer. Interestingly, both the promoter and the enhancers did not display H3K27me3 histone marker and Polycomb binding in CRC cells. Moreover, informatics analysis proved that the expression levels of PTX3 regulating miRNAs did not change between colon healthy and malignant cells. Thus, we suggest that DNA methylation is the only epigenetic mechanism regulating PTX3 gene expression in CRC, and this is dependent on silencing of enhancers. This is in line with recent results, demonstrating that the methylation of enhancers is responsible for silencing of target genes and it even plays a predominant role compared to promoter methylation [202,203].

The PTX3 promoter and long-range enhancer were active in CRC cells only upon demethylation treatment and in the presence of an inflammatory stimulus, which leads to the binding of transcription factors on regulatory elements. This confirmed our previous suggestions about the role of NF-kB in the regulation of the activity of PTX3 first enhancer. Indeed, several works demonstrated that both enhancer activity and loop stabilization depend on the binding of specific transcription factors [204,205].

The methylation analysis was then extended to CRC samples derived from patients with colon cancer at different stages (stage 1 to 4), as well as in adenoma. Both the enhancers were shown to be hypermethylated in tumors and the methylation started at the adenoma stage, suggesting that this is an early event in the natural history of colorectal cancer [206]. In particular, PTX3 intragenic enhancer was hypomethylated in the adenoma and hypermethylated in the advanced tumor stages, whereas the long-range enhancer was hypermethylated in adenoma stages and its methylation level decreased during tumor progression.

Our in vivo experiments demonstrated that PTX3 is activated in order to reduce cancer related inflammatory response. Given that the activation of PTX3 first enhancer depends on

inflammatory stimuli, its high levels of methylation can be necessary to repress and avoid the PTX3 activation in response to the cancer related inflammation. Thus, we infer that PTX3 methylation is not a casual event generated by the tumor mutagenesis, but it could be important events that favour the carcinogenesis itself [28, 198]. PTX3 enhancers were demonstrated to be hypermethylated in other tumours as well, both mesenchymal and epithelial, indicating that PTX3 silencing is a mechanism that might occur in several types of cancer.

Given the importance of the connection between inflammation and cancer [207-208], we suggest that PTX3 may act as an extrinsic oncosupressor gene related to cancer inflammation in several tumors.

References

1 Waddington, CH., Towards a Theoretical Biology. vol. 1: Prolegomena. Edinburgh: Edinburgh University Press, 1968 p. 1-32.

2 Bernstein BE, Meissner A, Lander ES, The mammalian epigenome. Cell, 2007, 128:669–681.

3 Lorenzen JM, Martino F, Thum T ,Epigenetic modifications in cardiovascular disease. Basic Res Cardiol, 2012, 107:245.

4 Wu JI, Lessard J, Crabtree GR,Understanding the words of chromatin regulation, Cell, 2009, 136:200–206.

5 Cathérine Dupont, Randall Armant, and Carol A. Brenner, Epigenetics: Definition, Mechanisms and Clinical Perspective, Semin Reprod Med., 2009 Sep;27(5):351-7.

6 Anna Portela, Manel Esteller, Epigenetic modifications and human disease, Nature biotechnology, 2010, 454, 766–770.

7 Rakyan, V. K., Down, T. A., Balding, D. J. & Beck, S. Epigenome-wide association studies for common human diseases. Nature Rev. Genet., 2011, 12, 529–541 .

8 *Cizhong Jiang and B. Franklin Pugh,* Nucleosome positioning and gene regulation: advances through genomics, Nature Rewies Genetics, 2009, 10(3):161-72.

9 Segal, E., and Widom, J., What controls nucleosome positions? Trends Genet, 2009, 25, 335–343 .

10 Valouev, A., Johnson, S.M., Boyd, S.D., Smith, C.L., Fire, A.Z., and Sidow, A. , Determinants of nucleosome organization in primary human cells. Nature, 2011, 474, 516–520 .

11 Reisman, D., Glaros, S., and Thompson, E.A., The SWI/SNF complex and cancer. Oncogene, 2009, 28, 1653–1668.

12 Wilson, B.G., and Roberts, C.W. , SWI/SNF nucleosome remodellers and cancer. Nat. Rev. Cancer, 2011, 11, 481–492.

13 Wang GG, Allis CD, Chi P., Chromatin remodeling and cancer, Part II: ATP-dependent chromatin remodeling., *Trends Mol Med.*, 2007, 13(9):373-80.

14 Mayes K, Qiu Z, Alhazmi A, Landry JW., ATP-dependent chromatin remodeling complexes as novel targets for cancer therapy, Adv Cancer Res., 2014, 121:183-233.

15 Klose RJ, Bird AP. Genomic DNA methylation: the mark and its mediators. *Trends Biochem Sci.,* 2006, 31:89-97.

16 Illingworth RS, Bird AP. CpG islands–'a rough guide.' *FEBS Lett.,* 2009, 583:1713-1720.

17 Nan X, Ng HH, Johnson CA, et al. Transcriptional repression by the methyl-CpGbinding protein MeCP2 involves a histone deacetylase complex. *Nature,* 1998, 393: 386-389.

18 Swain JL, Stewart TA, Leder P. Parental legacy determines methylation and expression of an autosomal transgene: a molecular mechanism for parental imprinting. *Cell,* 1987, 3:2023- 2036.

19 Mohandas T, Sparkes RS, Shapiro LJ. Reactivation of an inactive human X chromosome: evidence for X inactivation by DNA methylation. *Science.* 1981, 211: 393-396.

20 Wilson VL, Jones PA. DNA methylation decreases in aging but not in immortal cells. *Science,* 1983, Jun 3;220(4601):1055-7.

21 Bestor TH, Tycko B. Creation of genomic methylation patterns. *Nat Genet.* 1996, Apr;12(4):363-7.

22 Jones PA, Liang G. Rethinking how DNA methylation patterns are maintained. *Nat Rev Genet.,* 2009, Nov; 10(11): 805-11.

23 Jackson-Grusby, L. *et al.* Loss of genomic methylation causes p53-dependent apoptosis and epigenetic deregulation. Nature Genet, 2001, 27, 31–39 .

24 Chen, T. *et al.* Complete inactivation of DNMT1 leads to mitotic catastrophe in human cancer cells. *Nature Genet*, 2007, 39, 391–396 .

25 Bhutani, N. *et al.* Reprogramming towards pluripotency requires AID-dependent DNA demethylation. *Nature*, 2010, 463, 1042–1047.

26 Inoue, A. & Zhang, Y. Replication-dependent loss of 5-hydroxymethylcytosine in mouse preimplantation embryos. *Science*, 2011, 334, 194 .

27 Cortellino, S. *et al.* Thymine DNA glycosylase is essential for active DNA demethylation by linked deamination-base excision repair. *Cell*, 2011, 146, 67–79.

28 Baylin, S. B. & Jones, P. A. A decade of exploring the cancer epigenome — biological and translational implications. *Nature Rev. Cancer*, 2011, 11, 726–734.

29 Mills, A.A. Throwing the cancer switch: reciprocal roles of polycomb and trithorax proteins. Nat. Rev. Cancer, 2010, 10, 669–682.

30 Suganuma, T., and Workman, J.L.. Signals and combinatorial functions of histone modifications. Annu. Rev. Biochem., 2011, 80, 473–499.

31 Strahl BD, Allis CD. The language of covalent histone modifications. *Nature,* 2000, 403:41-45.

32 Struhl K. Histone acetylation and transcriptional regulatory mechanisms. *Genes Dev.*,1998, 12:599-606.

33 Yang XJ. The diverse superfamily of lysine acetyltransferases and their roles in leukemia and other diseases. *Nucleic Acids Res.,* 2004, Feb 11;32(3):959-76.

34 Utley RT, Ikeda K, Grant PA, Côté J, Steger DJ, Eberharter A, John S, Workman JL., Transcriptional activators direct histone acetyltransferase complexes to nucleosomes. *Nature, 1998,* 394:498-502 .

35 Yang XJ, Seto E. HATs and HDACs: from structure, function and regulation to novel strategies for therapy and prevention. *Oncogene,* 2007, 26:5310-5318.

36 Ashburner BP, Westerheide SD, Baldwin AS Jr. The p65 (RelA) subunit of NF-kappaB interacts with the histone deacetylase (HDAC) corepressors HDAC1 and HDAC2 to negatively regulate gene expression. *Mol Cell Biol,* 2001, 21:7065-7077.

37 Ikenoue T, Inoki K, Zhao B, Guan KL. PTEN acetylation modulates its interaction with PDZ domain. *Cancer Res.,* 2008, Sep 1;68(17):6908-12.

38 Vaziri H, Dessain SK, Ng Eaton E, et al. hSIR2(SIRT1) functions as an NAD-dependent p53 deacetylase. *Cell,*. 2001, Oct 19;107(2):149-59.

39 Greer, E.L., and Shi, Y. , Histone methylation: a dynamic mark in health, disease and inheritance. Nat. Rev. Genet., 2012, Apr 3;13(5):343-57.

40 Zhang K, Lin W, Latham JA, et al. The Set1methyltransferase opposes Ipl1 aurora kinase functions in chromosome segregation. *Cell.,* 2005, Sep 9;122(5):723-34.

41 Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell, 2004, 116:281–97.

42 Lau, N.C., Lim, L.P., Weinstein, E.G. & Bartel, D.P. An abundant class of tiny RNAs with probable regulatory roles in Caenorhabditis elegans. *Science* , 2001, 294, 858-62.

43 Lee, R.C. & Ambros, V. An extensive class of small RNAs in Caenorhabditis elegans. *Science*, 2001, 294, 862-4.

44 Wienholds E, Plasterk RH. MicroRNA function in animal development. FEBS Lett., 2005, 579:5911–22.

45 Sonkoly, E., and Pivarcsi, A., microRNAs in inflammation. Int. Rev. Immunol. 2009, 28, 535–561.

46 Recchiuti, A., Krishnamoorthy, S., Fredman, G., Chiang, N., and Serhan, C.N.. MicroRNAs in resolution of acute inflammation: identification of novel resolvin D1-miRNA circuits. FASEB J., 2011, 25, 544–560.

47 Ram Pyare Singh a, Israel Massachi a, Sudhir Manickavel a, Satendra Singh a, Nagesh P. Rao b, Sascha Hasan c, Deborah K. Mc Curdy d, Sherven Sharma f, David Wong e, Bevra H. Hahn a, Hamida Rehimi a The role of miRNA in inflammation and autoimmunity, Autoimmunity Reviews, 2013, Oct;12(12):1160-5.

48 Hwang HW, Mendell JT. MicroRNAs in cell proliferation, cell death, and tumorigenesis. Br J Cancer., 2006, 94:776–80.

49 Chekulaeva, M. & Filipowicz, W. Mechanisms of miRNA-mediated post-transcriptional regulation in animal cells. *Current opinion in cell biology* , 2009, 21, 452-60.

50 Filipowicz, W., Bhattacharyya, S.N. & Sonenberg, N. Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? *Nature reviews. Genetics*, 2008, 9, 102-14.

51 Inui, M., Martello, G. & Piccolo, S. MicroRNA control of signal transduction. *Nature reviews. Molecular cell biology* , 2010, 11, 252-63.

52 Liu, J., Valencia-Sanchez, M.A., Hannon, G.J. & Parker, R. MicroRNA-dependent localization of targeted mRNAs to mammalian P-bodies. *Nature cell biology*, 2005, 7, 719-23.

53 Jennifer L. Plank and Ann Dean, Enhancer Function: Mechanistic and Genome-Wide Insights Come Together, molcel., 2014, Jul 3;55(1):5-14.

54 *Daria Shlyueva, Gerald Stampfel and Alexander Stark,* Transcriptional enhancers: from properties to genome-wide predictions, nature review genetics, 2014, 54(1):180-192.

55 Dekker, J., Rippe, K., Dekker, M., and Kleckner, N. , Capturing chromosome conformation. Science, 2002, 295, 1306–1311.

56 Tolhuis, B., Palstra, R.J., Splinter, E., Grosveld, F., and de Laat, W., Looping and interaction between hypersensitive sites in the active b-globin locus. Mol. Cell, 2002, 10, 1453–1465.

57 Maston, G.A., Landt, S.G., Snyder, M., and Green, M.R.., Characterization of enhancer function from genome-wide analyses. Annu. Rev. Genomics Hum. Genet., 2012, 13, 29–57.

58 Ren, X., Siegel, R., Kim, U., and Roeder, R.G., Direct interactions of OCA-B and TFII-I regulate immunoglobulin heavy-chain gene transcription by facilitating enhancer-promoter communication. Mol. Cell, 2011, 42, 342–355 .

59 Liu, Z., Scannell, D.R., Eisen, M.B., and Tjian, R., Control of embryonic stem cell lineage commitment by core promoter factor, TAF3. Cell, 2011, 146, 720–731.

60 Koch, F., Fenouil, R., Gut, M., Cauchy, P., Albert, T.K., Zacarias-Cabeza, J., Spicuglia, S., de la Chapelle, A.L., Heidemann, M., Hintermair, C., Transcription initiation platforms and GTF recruitment at tissue-specific enhancers and promoters. Nat. Struct. Mol. Biol., 2011, Jul 17;18(8):956-63.

61 Liu, W., Ma, Q., Wong, K., Li, W., Ohgi, K., Zhang, J., Aggarwal, A.K., and Rosenfeld, M.G., Brd4 and JMJD6-associated anti-pause enhancers in regulation of transcriptional pause release. Cell 2013, Dec 19;155(7):1581-95.

62 Ørom, U.A., and Shiekhattar, R., Long noncoding RNAs usher in a new era in the biology of enhancers. Cell, 2013, 154, Issue 6, p1190–1193.

63 Sawarkar R, Paro R, Interpretation of developmental signaling at chromatin: the Polycomb perspective. Dev Cell, 2010, Nov 16;19(5):651-61.

64 Surface LE, Thornton SR, Boyer LA, Polycomb group proteins set the stage for early lineage commitment. Cell Stem Cell, 2010, Sep 3;7(3):288-98.

65 Mikkelsen TS, Ku M, Jaffe DB, Issac B, Lieberman E, Giannoukos G, Alvarez P, Brockman W, Kim TK, Koche RP, Lee W, Mendenhall E, O'Donovan A, Presser A, Russ C, Xie X, Meissner A, Wernig M, Jaenisch R, Nusbaum C, Lander ES, Bernstein BE., Genomewide maps of chromatin state in pluripotent and lineage-committed cells. Nature, 2007, 448, 553-560.

66 Rodolphe Taby, MD1; Jean-Pierre J. Issa, MD2, Cancer Epigenetics, CA CANCER J CLIN, 2010, 60:376-392.

67 Peng B, Hodge DR, Thomas SB, Cherry JM, Munroe DJ, Pompeia C, et al. Epigenetic silencing of the human nucleotide excision repair gene, hHR23B, in interleukin-6-responsive multiple myeloma KAS-6/1 cells. J Biol Chem, 2005, 11;280(6):4182-7.

68 Pompeia C, Hodge DR, Plass C, Wu YZ, Marquez VE, Kelley JA, et al. Microarray analysis of epigenetic silencing of gene expression in the KAS-6/1 multiple myeloma cell line. Cancer Res, 2004, May 15;64(10):3465-73.

69 Hodge DR, Peng B, Cherry JC, Hurt EM, Fox SD, Kelley JA, et al. Interleukin 6 supports the maintenance of p53 tumor suppressor gene promoter methylation. Cancer Res, 2005, Jun 1;65(11):4673-82.

70 Croonquist PA, Van Ness B. The polycomb group protein enhancer of zeste homolog 2 (EZH 2) is an oncogene that influences myeloma cell growth and the mutant ras phenotype. Oncogene, 2005, Sep 15;24(41):6269-80.

71 Winston Timp and Andrew P. Feinberg, Cancer as a dysregulated epigenome allowing cellular growth advantage at the expense of the host, Nat Rev Cancer, 2013, Jul;13(7):497-510.

72 Esteller M. Cancer epigenomics: DNA methylomes and histone-modification maps. *Nat Rev Genet.*, 2007, Apr;8(4):286-98.

73 Kleer CG, Cao Q, Varambally S, et al. EZH2 is a marker of aggressive breast cancer and promotes neoplastic transformation of breast epithelial cells. *Proc Natl Acad Sci USA.*, 2003, Sep 30;100(20):11606-11.

74 Martinez-Garcia E, Licht JD. Deregulation of H3K27 methylation in cancer. *Nat Genet.* 2010, Feb;42(2):100-1.

75 Peters AH, O'Carroll D, Scherthan H, et al. Loss of the Suv39h histone methyltransferases impairs mammalian heterochromatin and genome stability. *Cell.*, 2001, Nov 2;107(3):323-37.

76 Olive V, Jiang I, He L. mir-17-92, a cluster of miRNAs in the midst of the cancer network. *Int J Biochem Cell Biol*,. 2010, Aug;42(8):1348-54.

77 Weinhouse, S. Isozymes in cancer. *Cancer Res.*, 1971, Aug;31(8):1166-7.

78 Hansen, K. D. *et al.* Increased methylation variation in epigenetic domains across cancer types. *Nature Genet.*, 2011, Jun 26;43(8):768-75.

79 Figueroa, M. E. *et al.* Leukemic IDH1 and IDH2 mutations result in a hypermethylation phenotype, disrupt TET2 function, and impair hematopoietic differentiation. *Cancer Cell*, 2010,. 18(6):553-67

80 De Carvalho, D.D., Sharma, S., You, J.S., Su, S.F., Taberlay, P.C., Kelly, T.K., Yang, X., Liang, G., and Jones, P.A. , DNA methylation screening identifies driver epigenetic events of cancer cell survival. Cancer Cell, 2012, May 15;21(5):655-67.

81 Wu, S.C., and Zhang, Y. , Active DNA demethylation: many roads lead to Rome. Nat. Rev. Mol. Cell Biol, 2010, Sep;11(9):607-20.

82 Bhutani, N., Burns, D.M., and Blau, H.M. , DNA demethylation dynamics. Cell, 2011, Sep 16;146(6):866-72.

83 Ko, M., Huang, Y., Jankowska, A.M., Pape, U.J., Tahiliani, M., Bandukwala, H.S., An, J., Lamperti, E.D., Koh, K.P., Ganetzky, R., Impaired hydroxylation of 5-methylcytosine in myeloid cancers with mutant TET2, Nature, 2010, Dec 9;468(7325):839-43.

84 Livide G, Epistolato MC, Amenduni M, Disciglio V, Marozza A, Mencarelli MA, Toti P, Lazzi S, Hadjistilianou T, De Francesco S, D'Ambrosio A, Renieri A, Ariani F., Epigenetic and copy number variation analysis in retinoblastoma by MS-MLPA, Pathol Oncol Res. 2012, Jul;18(3):703-12.

85 Schwartzentruber, J. *et al.* Driver mutations in histone H3.3 and chromatin remodelling genes in paediatric glioblastoma. *Nature*, 2012, Jan 29;482(7384):226-31.

86 Krivtsov, A. V. & Armstrong, S. A. MLL translocations, histone modifications and leukaemia stem-cell development. *Nature Rev. Cancer* , 2007, Nov;7(11):823-33.

87 Hatziapostolou, M., and Iliopoulos, D. , Epigenetic aberrations during oncogenesis. Cell. Mol. Life Sci., 2011, 68(10):1681-702.

88 Esteller, M., Cancer epigenomics: DNA methylomes and histonemodification maps. Nat. Rev. Genet, 2007, Apr;8(4):286-98.

89 Ley, T.J., Ding, L., Walter, M.J., McLellan, M.D., Lamprecht, T., Larson, D.E., Kandoth, C., Payton, J.E., Baty, J., Welch, J., DNMT3A mutations in acute myeloid leukemia. N. Engl. J. Med, 2010, Dec 16;363(25):2424-33.

90 Balkwill, F. & Mantovani, A. Inflammation and cancer: back to Virchow? *Lancet* 357, 2001, Feb 17;357(9255):539-45.

91 Koehne, C. H. & Dubois, R. N. COX-2 inhibition and colorectal cancer. *Semin. Oncol.* , 2004, 31, 12–21.

92 Chan, A. T., Ogino, S. & Fuchs, C. S. Aspirin and the risk of colorectal cancer in relation to the expression of COX-2. *N. Engl. J. Med.*, 2007, May 24;356(21):2131-42.

93 Borrello MG, Alberti L, Fischer A, Degl'innocenti D, Ferrario C, Gariboldi M, Marchesi F, Allavena P, Greco A, Collini P, Pilotti S, Cassinelli G, Bressan P, Fugazzola L, Mantovani A, Pierotti MA., Induction of a proinflammatory program in normal human thyrocytes by the *RET/PTC1* oncogene. *Proc. Natl Acad. Sci. USA* , 2005, . Oct 11;102(41):14825-30.

94 Maciej M Markiewski, Robert A DeAngelis, Fabian Benencia, Salome K Ricklin-Lichtsteiner1, Anna Koutoulaki, Craig Gerard, George Coukos & John D Lambris, Modulation of the antitumor immune response by complement, Nature Immunology, 2008, Nov ;9(11):1225-35.

95 de Visser, K.E., Korets, L.V. & Coussens, L.M. Early neoplastic progression is complement independent. Neoplasia, 2004, Nov-Dec;6(6):768-76.

96 Huber-Lang M, Sarma JV, Zetoune FS, Rittirsch D, Neff TA, McGuire SR, Lambris JD, Warner RL, Flierl MA, Hoesel LM, Gebhard F, Younger JG, Drouin SM, Wetsel RA, Ward PA., Generation of C5a in the absence of C3: a new complement activation pathway. Nat. Med., 2006, Jun;12(6):682-7.

97 Shankaran V, Ikeda H, Bruce AT, White JM, Swanson PE, Old LJ, Schreiber RD., IFN-g and lymphocytes prevent primary tumour development and shape tumour immunogenicity. Nature , 2001, Apr 26;410(6832) 1107-11.

98 Daniel DS, Dai G, Singh CR, Lindsey DR, Smith AK, Dhandayuthapani S, Hunter RL Jr, Jagannath C. The reduced bactericidal function of complement C5-deficient murine macrophages is associated with defects in the synthesis and delivery of reactive oxygen radicals to mycobacterial phagosomes. J. Immunol., 2006, Oct 1;177(7):4688-98.

99 Borrello MG, Alberti L, Fischer A, Degl'innocenti D, Ferrario C, Gariboldi M, Marchesi F, Allavena P, Greco A, Collini P, Pilotti S, Cassinelli G, Bressan P, Fugazzola L, Mantovani A, Pierotti MA., Induction of a proinflammatory program in normal human thyrocytes by the *RET/PTC1* oncogene. *Proc. Natl Acad. Sci. USA* , 2005, Oct 11;102(41):14825-30.

100 Guerra C, Schuhmacher AJ, Cañamero M, Grippo PJ, Verdaguer L, Pérez-Gallego L, Dubus P, Sandgren EP, Barbacid M., Chronic pancreatitis is essential for induction of pancreatic ductal adenocarcinoma by K-Ras oncogenes in adult mice. *Cancer Cell* , 2007, Mar;11(3):291-302.

101 Sparmann, A. & Bar-Sagi, D. Ras-induced interleukin-8 expression plays a critical role in tumor growth and angiogenesis. *Cancer Cell* , 2004, . Mar;11(3):291-302

102 Sumimoto, H., Imabayashi, F., Iwata, T. & Kawakami, Y. The BRAF–MAPK signalling pathway is essential for cancer-immune evasion in human melanoma cells. *J. Exp. Med.*, 2006, Jul 10;203(7):1651-6.

103 Soucek L, Lawlor ER, Soto D, Shchors K, Swigart LB, Evan GI., Mast cells are required for angiogenesis and macroscopic expansion of Myc-induced pancreatic islet tumors. *Nature Med.*, 2007, Oct;13(10):1211-8.

104 Marx, J. Cancer immunology. Cancer's bulwark against immune attack: MDS cells. Science , 2008, Jan 11;319(5860):154-6.

105 Niculescu, F., Rus, H.G., Retegan, M. & Vlaicu, R. Persistent complement activation on tumor cells in breast cancer. Am. J. Pathol., 1992, May;140(5):1039-43.

106 Donin N, Jurianz K, Ziporen L, Schultz S, Kirschfink M, Fishelson Z., Complement resistance of human carcinoma cells depends on membrane regulatory proteins, protein kinases and sialic acid. Clin. Exp. Immunol. , 2003, Feb;131(2):254-63.

107 Biswas SK, Gangi L, Paul S, Schioppa T, Saccani A, Sironi M, Bottazzi B, Doni A, Vincenzo B, Pasqualini F, Vago L, Nebuloni M, Mantovani A, Sica A. A distinct and unique transcriptional program expressed by tumorassociated macrophages: defective NF-κB and enhanced IRF-3/STAT1 activation. *Blood* , 2006, Mar 1;107(5):2112-22.

108 Saccani A, Schioppa T, Porta C, Biswas SK, Nebuloni M, Vago L, Bottazzi B, Colombo MP, Mantovani A, Sica A., p50 nuclear factor-κB overexpression in tumor-associated macrophages inhibits M1 inflammatory responses and antitumor resistance. *Cancer Res.*, 2006, Dec 1;66(23):11432-40.

109 Lin KY, Guarnieri FG, Staveley-O'Carroll KF, Levitsky HI, August JT, Pardoll DM, Wu TC., Treatment of established tumors with a novel vaccine that enhances major histocompatibility class II presentation of tumor antigen. Cancer Res., 1996, Jan 1;56(1):21-6.

110 Bromberg JF, Wrzeszczynska MH, Devgan G, Zhao Y, Pestell RG, Albanese C, Darnell JE Jr., Stat3 as an oncogene. *Cell*, 1999, Aug 6;98(3):295-303.

111 Kortylewski M, Kujawski M, Wang T, Wei S, Zhang S, Pilon-Thomas S, Niu G, Kay H, Mulé J, Kerr WG, Jove R, Pardoll D, Yu H., Inhibiting Stat3 signaling in the hematopoietic system elicits multicomponent antitumor immunity. *Nature Med.*, 2005, Dec;11(12):1314-21.

112 Shchors K, Shchors E, Rostker F, Lawlor ER, Brown-Swigart L, Evan GI., The Myc-dependent angiogenic switch in tumors is mediated by interleukin 1beta. Genes Dev., 2006, Sep 15;20(18):2527-38.

113 Tu S, Bhagat G, Cui G, Takaishi S, Kurt-Jones EA, Rickman B, Betz KS, Penz-Oesterreicher M, Bjorkdahl O, Fox JG, Wang TC., Overexpression of interleukin-1beta induces gastric inflammation and cancer and mobilizes myeloid-derived suppressor cells in mice. Cancer Cell., 2008, Nov 4;14(5):408-19.

114 Krelin Y, Voronov E, Dotan S, Elkabets M, Reich E, Fogel M, Huszar M, Iwakura Y, Segal S, Dinarello CA, Apte RN., Interleukin-1beta-driven inflammation promotes the development and invasiveness of chemical carcinogen-induced tumors. Cancer Res., 2007, Feb 1;67(3):1062-71.

115 Mantovani A, Allavena P, Sica A, Balkwill F. Cancer-related inflammation. Nature, 2008, Jul 24;454(7203):436-44.

116 Balkwill,F., Cancer and the chemokine network. Nat. Rev. Cancer, 2004, Jul;4(7):540-50.

117 Balkwill FR, Mantovani A., Cancer-related inflammation: common themes and therapeutic opportunities, Semin Cancer Biol., 2012, Feb;22(1):33-40.

118 De Palma, M. *et al.* Tie2 identifies a hematopoietic lineage of proangiogenic monocytes required for tumor vessel formation and a mesenchymal population of pericyte progenitors. *Cancer Cell,* 2005, Sep;8(3):211-26.

119 Mantovani, A., Sozzani, S., Locati, M., Allavena, P. & Sica, A. Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. *Trends Immuno.*, 2002, Nov;23(11):549-55.

120 Condeelis J, Pollard JW. Macrophages: obligate partners for tumor cell migration, invasion, and metastasis. Cell., 2006, Jan 27;124(2):263-6.

121 Laoui D, Movahedi K, Van Overmeire E, Van den Bossche J, Schouppe E, Mommer C, Nikolaou A, Morias Y, De Baetselier P, Van Ginderachter JA., Tumor-associated macrophages in breast cancer: distinct subsets, distinct functions. Int J Dev Biol., 2011, 55(7-9):861-7.

122 Murdoch C, Muthana M, Coffelt SB, Lewis CE., The role of myeloid cells in the promotion of tumour angiogenesis. Nat. Rev. Cancer, 2008, 618-31.

123 Fischer, C. et al. Anti-PlGF inhibits growth of VEGFR-inhibitor-resistant tumors without affecting healthy vessels. Cell , 2007, 131, 463–475.

124 Kaplan RN, Riba RD, Zacharoulis S, Bramley AH, Vincent L, Costa C, MacDonald DD, Jin DK, Shido K, Kerns SA, Zhu Z, Hicklin D, Wu Y, Port JL, Altorki N, Port ER, Ruggero D, Shmelkov SV, Jensen KK, Rafii S, Lyden D., VEGFR1-positive haematopoietic bone marrow progenitors initiate the pre-metastatic niche, Nature, 2005, 438, 820–827.

125 Shojaei F, Wu X, Zhong C, Yu L, Liang XH, Yao J, Blanchard D, Bais C, Peale FV, van Bruggen N, Ho C, Ross J, Tan M, Carano RA, Meng YG, Ferrara N., Bv8 regulates myeloid-cell-dependent tumour angiogenesis, Nature, 2007, Dec 6;450(7171):825-31.

126 Lewis, C. E., De Palma, M. & Naldini, L. Tie2-expressing monocytes and tumor angiogenesis: regulation by hypoxia and angiopoietin 2. *Cancer Res.*,2007, Sep 15;67(18):8429-32.

127 Sozzani, S., Rusnati, M., Riboldi, E., Mitola, S. & Presta, M. Dendritic cell-endothelial cell cross-talk in angiogenesis. *Trends Immunol.*,2007, Sep;28(9):385-92.

128 Gavin P. Dunn, Allen T. Bruce, Hiroaki Ikeda, Lloyd J. Old & Robert D. Schreiber ,Cancer immunoediting: from immunosurveillance to tumor escape, *Nature Immunology* , 2002, 3, 991 - 998.

129 Salcedo R, Cataisson C, Hasan U, Yuspa SH, Trinchieri G., MyD88 and its divergent toll in carcinogenesis. Trends Immunol., 2013, Aug;34(8):379-89.

130 Sica, A. & Bronte, V. Altered macrophage differentiation and immune dysfunction in tumor development. *J. Clin. Invest.*, 2007, May;117(5):1155-66.

131 Lee, A. H. *et al.* Different patterns of inflammation and prognosis in invasive carcinoma of the breast. *Histopathology*, 2006, May;48(6):692-701.

132 Klintrup, K. *et al.* Inflammation and prognosis in colorectal cancer. *Eur. J. Cancer*, 2005, Nov;41(17):2645-54.

133 Cai, T. *et al.* Prognostic role of the tumor-associated tissue inflammatory reaction in transitional bladder cell carcinoma. *Oncol. Rep.*, 2006, Nov;41(17):2645-54.

134 Kirkwood, J. M. *et al.* Interferon alfa-2b adjuvant therapy of high-risk resected cutaneous melanoma: the Eastern Cooperative Oncology Group Trial EST 1684. *J. Clin. Oncol.*, 1996, Aug;16(2):329-34.

135 Rohatiner, A. Z. *et al.* Meta-analysis to evaluate the role of interferon in follicular lymphoma. *J. Clin. Oncol.*, 2005, 1996 Jan;14(1):7-17.

136 Garlanda, C., Bottazzi, B., Bastone, A. & Mantovani, A. Pentraxins at the crossroads between innate immunity, inflammation, matrix deposition, and female fertility. Annual review of immunology, 2005, Apr 1;23(10):2215-23.

137 Breviario F, d'Aniello EM, Golay J, Peri G, Bottazzi B, Bairoch A, Saccone S, Marzella R, Predazzi V, Rocchi M, Della Valle G, Dejana E, Mantovani A, Introna M: Interleukin-1-inducible genes in endothelial cells. Cloning of a new gene related to c-reactive protein and serum amyloid p component. J Biol Chem, 1992, 267:22190-22197.

138 Lee GW, Lee TH, Vilcek J: Tsg-14, a tumor necrosis factor- and il-1-inducible protein, is a novel member of the pentaxin family of acute phase proteins. J Immunol, 1993, 150:1804-1812.

139 Basile A, Sica A, d'Aniello E, Breviario F, Garrido G, Castellano M, Mantovani A, Introna M: Characterization of the promoter for the human long pentraxin ptx3. Role of nf-kappab in tumor necrosis factor-alpha and interleukin-1beta regulation. J Biol Chem, 1997, 272:8172-8178.

140 Altmeyer A, Klampfer L, Goodman AR, Vilcek J: Promoter structure and transcriptional activation of the murine tsg-14 gene encoding a tumor necrosis factor/interleukin-1-inducible pentraxin protein. J Biol Chem, 1995, 270:25584-25590.

141 Han B, Mura M, Andrade CF, Okutani D, Lodyga M, dos Santos CC, Keshavjee S, Matthay M, Liu M: Tnfalpha-induced long pentraxin ptx3 expression in human lung epithelial cells via jnk. J Immunol, 2005, 175:8303-8311.

142 Norata GD, Marchesi P, Pirillo A, Uboldi P, Chiesa G, Maina V, Garlanda C, Mantovani A, Catapano AL: Long pentraxin 3, a key component of innate immunity, is modulated by high-density lipoproteins in endothelial cells. Arterioscler Thromb Vasc Biol, 2008, 28:925-931.

143 Bottazzi B, Vouret-Craviari V, Bastone A, De Gioia L, Matteucci C, Peri G, Spreafico F, Pausa M, D'Ettorre C, Gianazza E, Tagliabue A, Salmona M, Tedesco F, Introna M, Mantovani A: Multimer

formation and ligand recognition by the long pentraxin ptx3. Similarities and differences with the short pentraxins c-reactive protein and serum amyloid p component. J Biol Chem, 1997, 272:32817-32823.

144 Inforzato A, Peri G, Doni A, Garlanda C, Mantovani A, Bastone A, Carpentieri A, Amoresano A, Pucci P, Roos A, Daha MR, Vincenti S, Gallo G, Carminati P, De Santis R, Salvatori G: Structure and function of the long pentraxin ptx3 glycosidic moiety: Fine-tuning of the interaction with c1q and complement activation. Biochemistry, 2006, 45:11540-11551.

145 Inforzato A, Rivieccio V, Morreale AP, Bastone A, Salustri A, Scarchilli L, Verdoliva A, Vincenti S, Gallo G, Chiapparino C, Pacello L, Nucera E, Serlupi-Crescenzi O, Day AJ, Bottazzi B, Mantovani A, De Santis R, Salvatori G: Structural characterization of ptx3 disulfide bond network and its multimeric status in cumulus matrix organization. J Biol Chem, 2008, 283:10147-10161.

146 Doni A, Peri G, Chieppa M, Allavena P, Pasqualini F, Vago L, Romani L, Garlanda C, Mantovani A: Production of the soluble pattern recognition receptor ptx3 by myeloid, but not plasmacytoid, dendritic cells. Eur J Immunol, 2003, 33:2886-2893.

147 Nauta AJ, de Haij S, Bottazzi B, Mantovani A, Borrias MC, Aten J, Rastaldi MP, Daha MR, van Kooten C, Roos A: Human renal epithelial cells produce the long pentraxin ptx3. Kidney Int 2005, 67:543-553.

148 Goodman AR, Levy DE, Reis LF, Vilcek J: Differential regulation of tsg-14 expression in murine fibroblasts and peritoneal macrophages. J Leukoc Biol, 2000, 67:387-395.

149 Bottazzi B, Garlanda C, Cotena A, Moalli F, Jaillon S, Deban L, Mantovani A: The long pentraxin ptx3 as a prototypic humoral pattern recognition receptor: Interplay with cellular innate immunity. Immunol Rev, 2009, 227:9-18.

150 Polentarutti N, Picardi G, Basile A, Cenzuales S, Rivolta A, Matteucci C, Peri G, Mantovani A, Introna M: Interferon-gamma inhibits expression of the long pentraxin ptx3 in human monocytes. Eur J Immunol, 1998, 28:496-501.

151 Doni A, Michela M, Bottazzi B, Peri G, Valentino S, Polentarutti N, Garlanda C, Mantovani A: Regulation of ptx3, a key component of humoral innate immunity in human dendritic cells: Stimulation by il-10 and inhibition by ifn-gamma. J Leukoc Biol, 2006, 79:797-802.

152 Jaillon S, Peri G, Delneste Y, Fremaux I, Doni A, Moalli F, Garlanda C, Romani L, Gascan H, Bellocchio S, Bozza S, Cassatella MA, Jeannin P, Mantovani A: The humoral pattern recognition receptor ptx3 is stored in neutrophil granules and localizes in extracellular traps. J Exp Med, 2007, 204:793-804.

153 Jaillon S, Moalli F, Ragnarsdottir B, Bonavita E, Puthia M, Riva F, Barbati E, Nebuloni M, Cvetko Krajinovic L, Markotic A, Valentino S, Doni A, Tartari S, Graziani G, Montanelli A, Delneste Y, Svanborg C, Garlanda C, Mantovani A., The humoral pattern recognition molecule PTX3 is a key component of innate immunity against urinary tract infection. Immunity, 2014, Apr 17;40(4):621-32.

154 Nauta AJ, Bottazzi B, Mantovani A, Salvatori G, Kishore U, Schwaeble WJ, Gingras AR, Tzima S, Vivanco F, Egido J, Tijsma O, Hack EC, Daha MR, Roos A: Biochemical and functional characterization of the interaction between pentraxin 3 and c1q. Eur J Immunol, 2003, 33:465-473.

155 Deban L, Jarva H, Lehtinen MJ, Bottazzi B, Bastone A, Doni A, Jokiranta TS, Mantovani A, Meri S: Binding of the long pentraxin ptx3 to factor h: Interacting domains and function in the regulation of complement activation. J Immunol, 2008, 181:8433-8440.

156 Braunschweig A, Jozsi M: Human pentraxin 3 binds to the complement regulator c4b-binding protein. PLoS One, 2011, 6:e23991.

157 Moalli F, Paroni M, Veliz Rodriguez T, Riva F, Polentarutti N, Bottazzi B, Valentino S, Mantero S, Nebuloni M, Mantovani A, Bragonzi A, Garlanda C: The therapeutic potential of the humoral pattern recognition molecule ptx3 in chronic lung infection caused by pseudomonas aeruginosa. J Immunol, 2011, 186:5425-5434.

158 Diniz SN, Nomizo R, Cisalpino PS, Teixeira MM, Brown GD, Mantovani A, Gordon S, Reis LF, Dias AA: Ptx3 function as an opsonin for the dectin-1-dependent internalization of zymosan by macrophages. J Leukoc Biol, 2004, 75:649-656.

159 Garlanda C, Hirsch E, Bozza S, Salustri A, De Acetis M, Nota R, Maccagno A, Riva F, Bottazzi B, Peri G, Doni A, Vago L, Botto M, De Santis R, Carminati P, Siracusa G, Altruda F, Vecchi A, Romani L, Mantovani A: Non-redundant role of the long pentraxin ptx3 in anti-fungal innate immune response. Nature, 2002, 420:182-186.

160 Bozza S, Bistoni F, Gaziano R, Pitzurra L, Zelante T, Bonifazi P, Perruccio K, Bellocchio S, Neri M, Iorio AM, Salvatori G, De Santis R, Calvitti M, Doni A, Garlanda C, Mantovani A, Romani L: Pentraxin 3 protects from mcmv infection and reactivation through tlr sensing pathways leading to irf3 activation. Blood, 2006, 108:3387-3396.

161 Reading PC, Bozza S, Gilbertson B, Tate M, Moretti S, Job ER, Crouch EC, Brooks AG, Brown LE, Bottazzi B, Romani L, Mantovani A: Antiviral activity of the long chain pentraxin ptx3 against influenza viruses. J Immunol, 2008, 180:3391-3398.

162 Gaziano R, Bozza S, Bellocchio S, Perruccio K, Montagnoli C, Pitzurra L, Salvatori G, De Santis R, Carminati P, Mantovani A, Romani L: Anti-aspergillus fumigatus efficacy of pentraxin 3 alone and in combination with antifungals. Antimicrob Agents Chemother 2004, Nov;48(11):4414-21.

163 Moalli F, Doni A, Deban L, Zelante T, Zagarella S, Bottazzi B, Romani L, Mantovani A, Garlanda C: Role of complement and fc(Altmeyer, Klampfer et al.) receptors in the protective activity of the long pentraxin ptx3 against aspergillus fumigatus. Blood 2010, 116:5170-5180.

164 Lu J, Marnell LL, Marjon KD, Mold C, Du Clos TW, Sun PD: Structural recognition and functional activation of fcgammar by innate pentraxins. Nature 2008, 456:989-992.

165 Job ER, Deng YM, Tate MD, Bottazzi B, Crouch EC, Dean MM, Mantovani A, Brooks AG, Reading PC: Pandemic h1n1 influenza a viruses are resistant to the antiviral activities of innate immune proteins of the collectin and pentraxin superfamilies. J Immunol 2010, 185:4284-4291.

166 Chiarini M, Sabelli C, Melotti P, Garlanda C, Savoldi G, Mazza C, Padoan R, Plebani A, Mantovani A, Notarangelo LD, Assael BM, Badolato R: Ptx3 genetic variations affect the risk of pseudomonas aeruginosa airway colonization in cystic fibrosis patients. Genes and immunity 2010, 11:665-670.

167 Olesen R, Wejse C, Velez DR, Bisseye C, Sodemann M, Aaby P, Rabna P, Worwui A, Chapman H, Diatta M, Adegbola RA, Hill PC, Ostergaard L, Williams SM, Sirugo G: Dc-sign (cd209), pentraxin 3 and vitamin d receptor gene variants associate with pulmonary tuberculosis risk in west africans. Genes and immunity 2007, 8:456-467.

168 Cunha C, Aversa F, Lacerda JF, Busca A, Kurzai O, Grube M, Loffler J, Maertens JA, Bell AS, Inforzato A, Barbati E, Almeida B, Santos e Sousa P, Barbui A, Potenza L, Caira M, Rodrigues F,

Salvatori G, Pagano L, Luppi M, Mantovani A, Velardi A, Romani L, Carvalho A: Genetic ptx3 deficiency and aspergillosis in stem-cell transplantation. N Engl J Med 2014, 370:421-432.

169 Barbati E, Specchia C, Villella M, Rossi ML, Barlera S, Bottazzi B, Crociati L, d'Arienzo C, Fanelli R, Garlanda C, Gori F, Mango R, Mantovani A, Merla G, Nicolis EB, Pietri S, Presbitero P, Sudo Y, Villella A, Franzosi MG: Influence of pentraxin 3 (ptx3) genetic variants on myocardial infarction risk and ptx3 plasma levels. PLoS One 2012, 7:e53030.

170 Diamond JM, Meyer NJ, Feng R, Rushefski M, Lederer DJ, Kawut SM, Lee JC, Cantu E, Shah RJ, Lama VN, Bhorade S, Crespo M, Demissie E, Sonett J, Wille K, Orens J, Weinacker A, Weill D, Arcasoy S, Shah PD, Belperio JA, Wilkes D, Ware LB, Palmer SM, Christie JD; Lung Transplant Outcomes Group, Variation in PTX3 is associated with primary graft dysfunction after lung transplantation, Am J Respir Crit Care Med. 2012, Sep 15;186(6):546-5 .

171 Deban L, Jaillon S, Garlanda C, Bottazzi B, Mantovani A: Pentraxins in innate immunity: Lessons from ptx3. Cell Tissue Res 2010, Jan;343(1):237-49.

172 Lech M, Römmele C, Gröbmayr R, Eka Susanti H, Kulkarni OP, Wang S, Gröne HJ, Uhl B, Reichel C, Krombach F, Garlanda C, Mantovani A, Anders HJ Endogenous and exogenous pentraxin-3 limits postischemic acute and chronic kidney injury, Kidney Int. 2013, 83:647-661.

173 Mantovani A, Valentino S, Gentile S, Inforzato A, Bottazzi B, Garlanda C: The long pentraxin ptx3: A paradigm for humoral pattern recognition molecules. Ann N Y Acad Sci 2013, May;1285:1-14 .

174 Souza DG, Amaral FA, Fagundes CT, Coelho FM, Arantes RM, Sousa LP, Matzuk MM, Garlanda C, Mantovani A, Dias AA, Teixeira MM: The long pentraxin ptx3 is crucial for tissue inflammation after intestinal ischemia and reperfusion in mice. Am J Pathol 2009, 174:1309-1318.

175 Salustri A, Garlanda C, Hirsch E, De Acetis M, Maccagno A, Bottazzi B, Doni A, Bastone A, Mantovani G, Beck Peccoz P, Salvatori G, Mahoney DJ, Day AJ, Siracusa G, Romani L, Mantovani A: Ptx3 plays a key role in the organization of the cumulus oophorus extracellular matrix and in in vivo fertilization. Development 2004, 131:1577-1586.

176 Scarchilli L, Camaioni A, Bottazzi B, Negri V, Doni A, Deban L, Bastone A, Salvatori G, Mantovani A, Siracusa G, Salustri A: Ptx3 interacts with inter-alpha-trypsin inhibitor: Implications for hyaluronan organization and cumulus oophorus expansion. J Biol Chem 2007, 282:30161-30170.

177 Inforzato A, Baldock C, Jowitt TA, Holmes DF, Lindstedt R, Marcellini M, Rivieccio V, Briggs DC, Kadler KE, Verdoliva A, Bottazzi B, Mantovani A, Salvatori G, Day AJ. The angiogenic inhibitor long pentraxin PTX3 forms an asymmetric octamer with two binding sites for FGF2. J Biol Chem. 2010, Jun 4;285(23):17681-92.

178 Ronca R, Di Salle E, Giacomini A, Leali D, Alessi P, Coltrini D, Ravelli C, Matarazzo S, Ribatti D, Vermi W, Presta M: Long pentraxin-3 inhibits epithelial-mesenchymal transition in melanoma cells, Mol Cancer Ther, 2013, Dec;12(12):2760-71.

179 Ronca R, Alessi P, Coltrini D, Di Salle E, Giacomini A, Leali D, Corsini M, Belleri M, Tobia C, Garlanda C, Bonomi E, Tardanico R, Vermi W, Presta M: Long pentraxin-3 as an epithelial-stromal fibroblast growth factor-targeting inhibitor in prostate cancer. J Pathol, 2013, 12:2760-2771.

180 Jun-Xiong Wang et all. Aberrant methylation of the 3q25 tumor suppressor gene PTX3 in human esophageal squamous cell carcinoma, *World J Gastroenterol*, *2011*, October 7; 17(37): 4225-4230.

181 Locatelli M[1], Ferrero S, Martinelli Boneschi F, Boiocchi L, Zavanone M, Maria Gaini S, Bello L, Valentino S, Barbati E, Nebuloni M, Mantovani A, Garlanda C.; The long pentraxin PTX3 as a correlate of cancer-related inflammation and prognosis of malignancy in gliomas., J Neuroimmunol. 2013, Jul 15;260(1-2):99-106.

182 Eleftherios P. Diamandis, et all. Pentraxin-3 Is a Novel Biomarker of Lung Carcinoma. Clin Cancer Res, 2011, 17(8); 2395–9.

183 Barretina J, Taylor BS, Banerji S, Ramos AH, Lagos-Quintana M, Decarolis PL, Shah K, Socci ND, Weir BA, Ho A, Chiang DY, Reva B, Mermel CH, Getz G, Antipin Y, Beroukhim R, Major JE, Hatton C, Nicoletti R, Hanna M, Sharpe T, Fennell TJ, Cibulskis K, Onofrio RC, Saito T, Shukla N, Lau C, Nelander S, Silver SJ, Sougnez C, Viale A, Winckler W, Maki RG, Garraway LA, Lash A, Greulich H, Root DE, Sellers WR, Schwartz GK, Antonescu CR, Lander ES, Varmus HE, Ladanyi M, Sander C, Meyerson M, Singer S., Subtype-specific genomic alterations define new targets for soft-tissue sarcoma therapy", Nature Genetic, 2010, Aug;42(8):715-21.

184 Riker AI, Enkemann SA, Fodstad O, Liu S, Ren S, Morris C, Xi Y, Howell P, Metge B, Samant RS, Shevde LA, Li W, Eschrich S, Daud A, Ju J, Matta J., The gene expression profiles of primary and

metastatic melanoma yields a transition point of tumor progression and metastasis. BMC Med Genomics, 2008, Apr 28;1:13.

185 Hong Y, Downey T, Eu KW, Koh PK, Cheah PY. A 'metastasis-prone' signature for early-stage mismatch-repair proficient sporadic colorectal cancer patients and its implications for possible therapeutics. Clin Exp Metastasis, 2010, Feb;27(2):83-90.

186 Akhtar-Zaidi B, Cowper-Sal-lari R, Corradin O, Saiakhova A, Bartels CF, Balasubramanian D, Myeroff L, Lutterbaugh J, Jarrar A, Kalady MF, Willis J, Moore JH, Tesar PJ, Laframboise T, Markowitz S, Lupien M, Scacheri PC, Epigenome enhancers profiling defines a signature of colon cancer, Science, 2012, May 11;336(6082):736-9 .

187 Creyghton MP, Cheng AW, Welstead GG, Kooistra T, Carey BW, Steine EJ, Hanna J, Lodato MA, Frampton GM, Sharp PA, Boyer LA, Young RA, Jaenisch R, H3k27Ac separates active from poised enhancers and predicts development state, Proc Natl Acad Sci U S A. 2010, 107(50):21931-6.

188 Heintzman ND, Hon GC, Hawkins RD, Kheradpour P, Stark A, Harp LF, Ye Z, Lee LK, Stuart RK, Ching CW, Ching KA, Antosiewicz-Bourget JE, Liu H, Zhang X, Green RD, Lobanenkov VV, Stewart R, Thomson JA, Crawford GE, Kellis M, Ren B., Histone modifications at human enhancers reflect global cell-type-specific gene expression , Nature, 2009, May 7;459(7243):108-12.

189 Rada-Iglesias A, Bajpai R, Swigut T, Brugmann SA, Flynn RA, Wysocka J., A unique chromatin signature uncovers early developmental enhancers in humans, , 2011, Feb 10;470(7333):279-83.

190 Visel A, Rubin EM, Pennacchio L.A., Genomics views of distant acting enhancers, Nature 2009, Sep 10;461(7261):199-205.

191 Ana Pombo: Modifications of RNA polymerase II are pivotal in regulating gene expression states, EMBO report 2009, 2009 Nov;10(11):1213-9.

192 Brookes E, de Santiago I, Hebenstreit D, Morris KJ, Carroll T, Xie SQ, Stock JK, Heidemann M, Eick D, Nozaki N, Kimura H, Ragoussis J, Teichmann SA, Pombo A., Polycomb associates genome-wide with a specific RNA polymerase II variant, and regulates metabolic genes in ESCs, Cell Stem Cell, 2012, Feb 3;10(2):157-70 .

193 Sun X, Sit A, Feinberg MW, Role of miR-181 family in regulating vascular inflammation and immunity, Trends Cardiovasc Med. 2014 Apr;24(3):105-12.

194 Taganov KD, NF-kappaB-dependent induction of microRNA miR-146, an inhibitor targeted to signaling proteins of innate immune responses. *Proc Natl Acad Sci U S A*, 2006, 103(33):12481-12486.

195 Tang B, Xiao B, Liu Z, Li N, Zhu ED, Li BS, Xie QH, Zhuang Y, Zou QM, Mao XH., Identification of MyD88 as a novel target of miR-155, involved in negative regulation of Helicobacter pylori-induced inflammation, FEBS Lett., 2010, 584(8):1481-1486.

196 Rudnicki A, Shivatzki S, Beyer LA, Takada Y, Raphael Y, Avraham KB, microRNA-224 regulates Pentraxin 3, a component of the humoral arm of innate immunity, in inner ear inflammation, Human Molecular Genetics, 2014, 15;23(12):3138-46.

197 Sandoval and Esteller, Cancer epigenomics: beyond genomics, Curr Opin Genet Dev, 2012, Feb;22(1):50-5.

198 Koch F, Fenouil R, Gut M, Cauchy P, Albert TK, et al. Transcription initiation platforms and GTF recruitment at tissue-specific enhancers and promoters. Nat Struct Mol Biol, 2011, Jul 17;18(8):956-6.

199 Szutorisz H, Dillon N, Tora L The role of enhancers as centres for general transcription factor recruitment. Trends Biochem Sci, 2005, Nov;30(11):593-9.

200 Stumpf M, Yue X, Schmitz S, Luche H, Reddy JK, et al. Specific erythroid-lineage defect in mice conditionally deficient for Mediator subunit Med1. Proc Natl Acad Sci U S A, 2010, Dec 14;107(50):21541-6.

201 Aran D, Hellman A., DNA methylation of transcriptional enhancers and cancer predisposition, Cell. 2013, Jul 3;154(1):11-3 .

202 Blattler A, Yao L, Witt H, Guo Y, Nicolet CM, Berman BP, Farnham PJ., Global loss of DNA methylation uncovers intronic enhancers in genes showing expression changes, Genome Biology 2014, Sep 20;15(9):469 .

203 Shlyueva D, Stampfel G, Stark A., Transcriptional enhancers: from properties to genome-wide predictions, *NATURE REVIEWS genetics, 2014*, Apr;15(4):272.

204 Plank JL, Dean A., Enhancer Function: Mechanistic and Genome-Wide Insights Come Together, Molecular Cell Review, 2014, Jul 3;55(1):5-14. .

205 Vogelstein, B., Papadopoulos, N., Velculescu, V.E., Zhou, S., Diaz, L.A., Jr., and Kinzler, K.W. Cancer genome landscapes, Science, 2013, Jul 3;55(1):5-14.

206 Coussens, L.M., Zitvogel, L., and Palucka, A.K. Neutralizing tumor-promoting chronic inflammation: a magic bullet? Science, 2013, Jan 18;339(6117): 286-91.

207 Grivennikov, S.I., Greten, F.R., and Karin, M. Immunity, inflammation, and cancer Cell. 2010, 140(6):883-99.

208 Hanahan, D., and Weinberg, R.A., Hallmarks of cancer: the next generation, Cell 2011, Mar 4;144(5):646-74.