University of Trás-os-Montes and Alto Douro

Disclosing the *FA-SAT* ncRNA role in cancer cells

Master's Dissertation in
Technologic, Comparative and Molecular Genetics

Diogo Ferreira Lucas

Advisors:

Professora Doutora Raquel Maria Garcia dos Santos Chaves

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Vila Real, 2022

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Vila Real, 2022

This master's dissertation was specifically prepared to obtain the MSc degree in Technologic, Comparative and Molecular Genetics and it complies with the scientific and technical norms required by the regulations of the University of Trás-os-Montes and Alto Douro.

The doctrines presented are solely the responsibility of the author. All non-original contributions have been identified, indicating the respective source.

"Para ser grande, sê inteiro: nada
Teu exagera ou exclui.

Sê todo em cada coisa. Põe quanto és
No mínimo que fazes.

Assim em cada lago a lua toda
Brilha, porque alta vive."

Fernando Pessoa, Odes de Ricardo Reis

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Abstract

Over the last years, satellite DNA (satDNA) sequences have attracted some attention from the scientific community, as important contributors to cell function and disease. *FA-SAT*, the major satDNA of the domestic cat genome, was originally discovered in this species, but it is known it is highly conserved and transcribed into non-coding RNA (*FA-SAT* ncRNA) in several Bilateria species, including humans. Earlier, the characterization of the ncRNA profile, in distinct cat cell lines, revealed the preservation of its nuclear location, with some differences in its distribution throughout the cell cycle: its expression was defined as cell-cycle dependent in non-tumor cells and the dysregulation of it was observed in the tumor ones. Moreover, the functions of this ncRNA were described in cat and human cells, having been stated its nuclear interaction with the PKM2 protein, to regulate the shift from cell proliferation to apoptosis.

With this work, we intended to perform the cellular characterization of these transcripts, for the first time, in humans. For that, we scrutinized the *FA-SAT* ncRNA profile in distinct human cell lines, analyzing and comparing its location, amount and appearance. Its behavior across the cell cycle was also evaluated in one of these cell lines. Then, we developed functional assays to unveil the mechanism by which the *FA-SAT* ncRNA/PKM2 complex can cross-talk between the mitogenic and apoptotic pathways, particularly, the P53 involvement in determining these phenotypes (both P53 wild-type-WT and null cells were used).

Our results revealed a similar nuclear location for the ncRNA in all the human cell lines analyzed, with some differences in its amount and appearance, depending on the cell type. A closer look at this ncRNA location in the HeLa cells showed that it was additionally located at the nucleolus (and its periphery). Regarding the analysis of its behavior along the cell division process of the HeLa cells, it was possible to detect *FA-SAT* transcripts in all the phases of the cell cycle (with major enrichment in the G1 phase and G1/S transition).

Concerning the *FA-SAT* functional assays, its knockdown resulted in decreased amounts of PKM2 nuclear protein (also its RNA) and in inhibition of cell proliferation (with *MYC* downregulation), both in P53 wild-type and null cells. Upon *FA-SAT* silencing, an apoptosis outcome was also verified in both cell lines, but the P53 accumulation was only observed in the P53 wild-type cells. Therefore, it was confirmed the *FA-SAT* ncRNA/PKM2 association at the nucleus of these human cells, to regulate the shift from cell proliferation to apoptosis, and it was stated the independence from the P53 status of these phenotypes.

In brief, this work represents an advance in knowledge, so it contributes to better understanding how the *FA-SAT* expression (and the *FA-SAT* ncRNA/PKM2 association) can modulate the cell-cycle progression/cell death, in the human cells. Additionally, it supports the comprehension of the largest fraction of our genomes (DNA repetitive sequences), as important functional elements responsible for the (dys)regulation of several cellular processes and diseases, such as cancer.

Keywords: *FA-SAT* ncRNA; human cancer cell lines; PKM2; mitogenic pathway; apoptosis; P53.

Resumo

Ao longo dos anos, as sequências de DNA satélite (satDNA) têm atraído a atenção da comunidade científica, como importantes contribuintes para a função celular e doença. *FA-SAT*, o principal satDNA do genoma do gato doméstico, foi originalmente descoberto nesta espécie, mas sabe-se que está altamente conservado e é transcrito sob a forma de RNA não-codificante (*FA-SAT* ncRNA) em várias espécies Bilateria, incluindo o Homem. Anteriormente, a caracterização do perfil deste ncRNA, em diferentes linhas celulares de gato, revelou a preservação da sua localização nuclear, existindo diferenças na sua distribuição ao longo do ciclo celular: a sua transcrição foi definida como dependente de ciclo em células não tumorais e a sua desregulação foi observada nas tumorais. Além disso, as funções deste ncRNA foram descritas em células de gato e humanas, tendo sido demonstrada a sua interação nuclear com a proteína PKM2, para regular a transição entre proliferação celular e apoptose.

Com este trabalho, pretendemos efetuar a caracterização celular destes transcritos, pela primeira vez, no Homem. Para isso, examinámos o perfil do *FA-SAT* ncRNA em diferentes linhas celulares humanas, analisando e comparando a sua localização, quantidade e aparência. O seu comportamento ao longo do ciclo celular também foi avaliado numa dessas linhas celulares. Seguidamente, desenvolvemos ensaios funcionais para desvendar o mecanismo pelo qual o complexo *FA-SAT* ncRNA/PKM2 consegue efetuar a comunicação entre as vias mitogénica e apoptótica, particularmente, o envolvimento de P53 na determinação desses fenótipos (foram utilizadas células P53 *wild-type*-WT e nulas).

Os nossos resultados revelaram uma localização nuclear semelhante do ncRNA em todas as linhas celulares humanas analisadas, existindo diferenças na sua quantidade e aparência, dependendo do tipo celular. Um olhar mais atento sobre a localização deste ncRNA nas células HeLa demostrou que este estava adicionalmente localizado no nucléolo (e na sua periferia). Acerca da análise do seu comportamento ao longo do processo de divisão celular das células HeLa, foi possível detetar transcritos *FA-SAT* em todas as fases do ciclo celular (com maior acumulação na fase G1 e transição G1/S).

Relativamente aos ensaios funcionais do *FA-SAT*, o seu silenciamento resultou na diminuição da quantidade de proteína nuclear PKM2 (também do seu RNA) e na inibição da proliferação celular (com subexpressão de *MYC*), tanto em células P53 WT como nulas. Após o silenciamento do *FA-SAT*, verificou-se também a ocorrência de apoptose nas duas linhas celulares, mas a acumulação de P53 foi observada apenas nas células P53 WT. Assim, foi

confirmada a associação *FA-SAT* ncRNA/PKM2 no núcleo dessas células humanas, para regular a transição entre proliferação celular e apoptose, e definiu-se que a determinação desses fenótipos era independente do estado de P53.

Em suma, este trabalho representa um avanço de conhecimento, uma vez que contribui para melhor compreender como é que a expressão de *FA-SAT* (e a associação *FA-SAT* ncRNA/PKM2) pode modular a progressão do ciclo celular/morte celular, nas células humanas. Adicionalmente, promove a compreensão da maior fração dos nossos genomas (sequências repetitivas de DNA), como elementos funcionais importantes e responsáveis pela (des)regulação de diversos processos celulares e doenças, como o cancro.

Palavras-chave: *FA-SAT* ncRNA; linhas celulares de cancro humano; PKM2; via mitogénica; apoptose; P53.

Publications and Communications

The data presented in this master's dissertation was used as a basis for the preparation of several papers to be published in international scientific journals with Referee. These results were also presented as oral and poster communications in national and international meetings.

Publications in international scientific journals with Referee (in preparation)

Lucas D, Ferreira D, Adega F, Chaves R. Decoding the most conserved satellite DNA ever described: *FA-SAT* ncRNA characterization, main interactors and roles in cell function and disease. *In preparation*.

Lucas D, Ferreira D, Adega F, Chaves R. Disclosing the *FA-SAT* ncRNA profile in human cancer cells: can its (dys)regulation be associated with some cancer features? *In preparation*.

Lucas D, Ferreira D, Adega F, Chaves R. *FA-SAT* ncRNA/PKM2 regulates the cell-cycle progression in human cancer cells: is the P53 status a determining factor? *In preparation*.

Oral communications

Lucas D, Ferreira D, Adega F, Chaves R (2021) *FA-SAT* ncRNA transcriptional characterization: can its distribution influence cancer features? II International Meeting of the Portuguese Society of Genetics. Vila Real, Portugal.

Lucas D, Ferreira D, Adega F, Chaves R (2021) Inducing the *FA-SAT* knockdown in human cancer cells - can the P53 status dictate different outcomes? II Jornadas de Biotecnologia para as Ciências da Saúde. Vila Real, Portugal.

Lucas D, Ferreira D, Adega F, Chaves R (2021) Comparing the *FA-SAT* ncRNA profile in different human cell lines: Is the expression of *FA-SAT* (dys)regulated in the cell cycle of HeLa cells? XIII Jornadas de Genética e Biotecnologia/III Jornadas Ibéricas de Genética Y Biotecnología. Vila Real, Portugal / Léon, Espanha.

Lucas D, Ferreira D, Adega F, Chaves R (2021) Insights on the molecular mechanism underlying the apoptosis phenotype of *FA-SAT* ncRNA/PKM2 complex disruption. Jornadas da Ciência-UTAD. Vila Real, Portugal.

Lucas D, Ferreira D, Adega F, Chaves R (2020) *FA-SAT* ncRNA depletion induces apoptosis – is it through p53 mediation? XII Genetics and Biotechnology Conference / II Genetics and Biotechnology Iberian Conference. Vila Real, Portugal / Léon, Espanha.

Poster communications

Ferreira D, Lucas D, Adega F, Chaves R (2021) Disclosing how *FA-SAT* ncRNA promotes the switch between cell proliferation and apoptosis. EMBO|EMBL Symposium: The Non-Coding Genome 2021. Heidelberg, Germany.

Lucas D, Ferreira D, Adega F, Chaves R (2021) Disclosing how *FA-SAT* ncRNA/PKM2 complex interferes with proliferative and apoptotic pathways and their key players. VI Jornadas da Bioquímica. Porto, Portugal.

Lucas D, Ferreira D, Adega F, Chaves R (2021) Cellular characterization of *FA-SAT* ncRNA in cancer cells. XXIII Encontro Nacional de Estudantes de Biologia (ENEB). Coimbra, Portugal.

Awards

Best Oral Communication Award (2021) II Jornadas de Biotecnologia para as Ciências da Saúde. Vila Real, Portugal.

Best Oral Communication Award in the Human Genetics & Biotechnology Category (2021) XIII Jornadas de Genética e Biotecnologia/III Jornadas Ibéricas de Genética Y Biotecnología. Vila Real, Portugal / Léon, Espanha.

Best Poster Award (2021) XXIII Encontro Nacional de Estudantes de Biologia (ENEB). Coimbra, Portugal.

Best Oral Communication Award (2021) Jornadas da Ciência-UTAD. Vila Real, Portugal.

Honorable Mention Award in the Oral Communication Category (2020) XII Genetics and Biotechnology Conference / II Genetics and Biotechnology Iberian Conference. Vila Real, Portugal / Léon, Espanha.

Index

List of Figures

List of Tables

Abbreviations

In this list, the word abbreviations that were used more than twice in the text are presented. The chemical symbols/formulas internationally recognized and contained in the IUPAC (International Union of Pure and Applied Chemistry) nomenclature were not included. Also, the abbreviations for genes and proteins were not here listed since they can be consulted in the nomenclature committees of each species.

DNA - Deoxyribonucleic acid
FBS - Fetal Bovine Serum
FISH - Fluorescent *in situ* Hybridization
FMT - Feline Mammary Tumor
IF - Immunofluorescence
LNA - Locked Nucleic Acid
ncRNA - Non-Coding RNA
RNA - Ribonucleic acid
RT - Room temperature
RT-qPCR - Reverse Transcriptase Quantitative Polymerase Chain Reaction
satDNA - Satellite DNA
satncRNA - Satellite Non-Coding RNA
SD – Standard Deviation
WT - Wild-type

Cell lines list

A549 - human lung tumor cell line (P53 wild-type)
FCAn - cat mammary non-tumor cell line
FkMTp - cat mammary tumor cell line
HeLa - human cervical tumor cell line
H1299 - human lung tumor cell line (P53 null)

Species list

FCA - *Felis catus*
HSA - *Homo sapiens*

CHAPTER I

General introduction

Review article:

Lucas D, Ferreira D, Adega F, Chaves R. Decoding the most conserved satellite DNA ever described: *FA-SAT* ncRNA characterization, main interactors and roles in cell function and disease. *In preparation*.

I.1. Satellite DNA, the dark matter of the genomes

Satellite DNAs (satDNAs) are a class of highly repetitive tandem sequences, being considered the main component of heterochromatin in eukaryotic genomes. The repetition of their monomeric units leads to the formation of long tandem arrays, at the (peri)centromeric and (sub)telomeric regions, or short tandem arrays, dispersed throughout the genome (Plohl et al. 2008; Plohl et al. 2012; Chaves et al. 2017).

Among the mechanisms that can explain the origin and evolution of satDNAs are the unequal crossing-over, the replication slippage and the rolling circle replication. They promote changes in the nucleotide sequence, monomer length, copy number or repetition complexity (Smith 1976; Charlesworth et al. 1994; Slamovits and Rossi 2002). The same satDNA can also present distinct distribution in the genomes (caused, for example, by rolling circle mechanism, with the migration of the extrachromosomal circular DNA to other regions of the genomes, or by the activity of some transposable elements, existing in the flanking regions of the satDNAs). For these reasons, generally, satDNAs are not well-conserved between distant taxa, making some satDNA families species-specific (Chaves et al. 2017).

Furthermore, due to its repetitive nature, complexity, high level of sequence polymorphism and rapid evolution (promoting differences in their copy number and distribution), there are some difficulties in the approach and experimental design for the study of satDNAs (reviewed in Ferreira et al. 2015; Louzada et al. 2020). For a long time, the information about this type of sequences remained scarce, with very few data in genome assemblies and sequence expression databases. Thus, the satDNAs were considered as junk DNA/dark matter of the genomes and it

was believed that its transcription was suppressed by the heterochromatin structure (Plohl et al. 2008; Ruiz-Ruano et al. 2016; Chaves et al. 2017).

Nevertheless, the increasing interest in studying the part of the transcriptomes that is not involved in protein coding, namely non-coding RNAs (ncRNAs), allowed the scientific community to realize that satDNA sequences could also be transcribed into small and long satellite non-coding RNAs (satncRNAs), being associated with important cellular functions (Ugarkovic 2005; Biscotti et al. 2015; Ferreira et al. 2015; Chaves et al. 2017; Ferreira et al. 2019a; Ferreira et al. 2019b).

The recent knowledge advances and the improvements in technologies such as DNA/RNA sequencing and computational biology, combined with other long-established methods (e.g., fluorescent *in situ* hybridization, FISH), have offered a new range of resources for studying satDNAs (and other repeats), with higher resolution and detail (reviewed in Weiss-Schneeweiss et al. 2015; Garrido-Ramos 2017; Louzada et al. 2020). Thereby, new insights regarding their composition, organization/distribution, abundance, variation, evolution and function have been established (Ruiz-Ruano et al. 2016; Belyayev et al. 2019; reviewed in Louzada et al. 2020), so we can begin to fully understand the largest fraction of our genome.

I.1.1. Satellite non-coding RNAs in cell function and disease

The evidence that satncRNAs could be involved in the regulation of several cellular processes and pathways promoted the interest in their study and resulted in their discovery in a variety of species (including vertebrates, invertebrates and plants), displaying high abundance, variability and multifunctionality (Ugarkovic 2005; Biscotti et al. 2015; Chaves et al. 2017; Ferreira 2018).

These transcripts showed differential expression in distinct cell states or types, being associated with several cellular functions/mechanisms, such as chromatin structure and function, chromosome pairing and segregation, gene expression modeling, cell proliferation, apoptosis, response to stresses, among others (reviewed in Ferreira et al. 2015). Additionally, the dysregulation of their transcription was related to specific diseases, including cancer (Figure I.1) (Enukashvily and Ponomartsev 2013; Saha et al. 2019).

In fact, several studies have already reported the amplification (Bersani et al. 2015) or hypomethylation (Walton et al. 2014) of different satDNAs and, consequently, their overexpression in a variety of cancers (Wilson et al. 2007; Ting et al. 2011; Saksouk et al. 2015). Due to the various roles played by satncRNAs in the cellular context, its aberrant

expression could be the cause of the genomic instability observed in some tumor cells: alterations in the levels of specific satncRNAs could lead to incorrect chromosome segregation, aneuploidy, chromatin decondensation or DNA breaks and rearrangements, resulting in genomic instability and contributing to the setting of malignancy (Sana et al. 2012; Saksouk et al. 2015; Saha et al. 2019).

On the other hand, it is also possible that the overexpression of satDNAs is a consequence of the genomic instability previously existing in the cells: the alteration of the levels of some satncRNAs can result from the activity alterations of tumor suppressor genes, such as *P53* (Leonova et al. 2013) or *BRCA1* (Zhu et al. 2018).

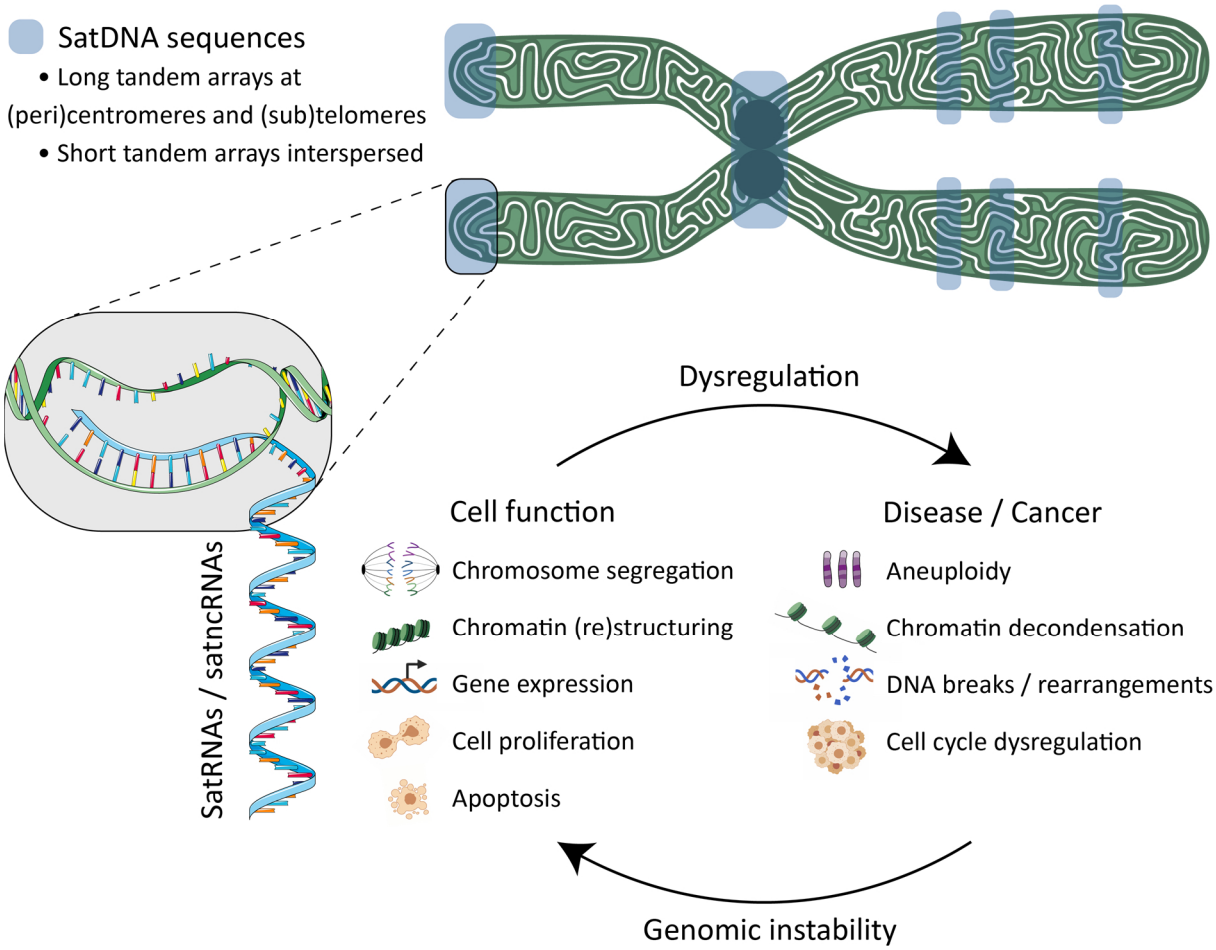

Figure I.1 - SatDNAs distribution/organization and some functions assigned to their transcripts (satRNAs). SatDNAs repetition can result either in long tandem arrays at the (peri)centromeric and (sub)telomeric regions or in short tandem arrays that disperse through the genome. Their transcription originates satRNAs/satncRNAs that play crucial roles in cell function and whose dysregulation can lead to diseases, such as cancer.

Notwithstanding, the correlation between the expression of various satDNAs and distinct features of tumor cells is evident. The exploration of this relation and the comparison of their

transcription status in a variety of tumor and non-tumor cells will allow us to elucidate the satncRNAs contribution to different cell processes and phenotypes, to determine their value as cancer biomarkers and to possibly define new diagnostic, prognostic and therapeutic tools, based on their evaluation (Ferreira 2018).

I.2. *FA-SAT* as a functional satellite DNA sequence

FA-SAT is the major satDNA sequence in the domestic cat *(Felis catus*, FCA) genome, representing about 1-2% of it. It was characterized for the first time by Fanning, as comprising a 483bp monomer tandemly repeated, with 64% of G+C (Fanning 1987). This monomer is organized into two different fractions: 30% of it is composed of a hexanucleotide motif (TAACCC), repeated imperfectly about 25 times (with some variations in motif sequence); 70% comprehends a unique G+C rich sequence, with low or no internal homology (Fanning 1987). In its sequence, it is also possible to identify the presence of a CpG island (Chaves et al. 2017).

FA-SAT is mainly located at the telomeres, but also at the centromeres or interstitially, in the cat chromosomes (Modi et al. 1988; Santos et al. 2004), being deemed as the major component of the heterochromatin in this genome. It presents a variable distribution/amount amongst the different felid species and the different chromosomes of the same species (Fanning et al. 1988), with some of the cat chromosomes showing no hybridization signals in the *FA-SAT* physical mapping by FISH, performed by Santos et al. (2004). Some of the main features of *FA-SAT* are represented in Figure I.2.

In 2009, Pontius and O'Brien, using data from an assembly of the whole-genome shotgun sequence of the cat genome, confirmed that *FA-SAT* represents about 2% of its genome, being repeated more than 100000 times. They also inferred that the low intersequence variability of *FA-SAT* DNA could be due to its high G+C content and to the presence of the TAACCC motif, repeated about 25 times in each monomer (Fanning 1987; Pontius and O'Brien 2009). Tamazian et al. (2014) detected the presence of different variants of the *FA-SAT* DNA in the cat genome assembly. However, these variants showed to share around 90% of similarity, agreeing with the information reported by Pontius and O'Brien (2009).

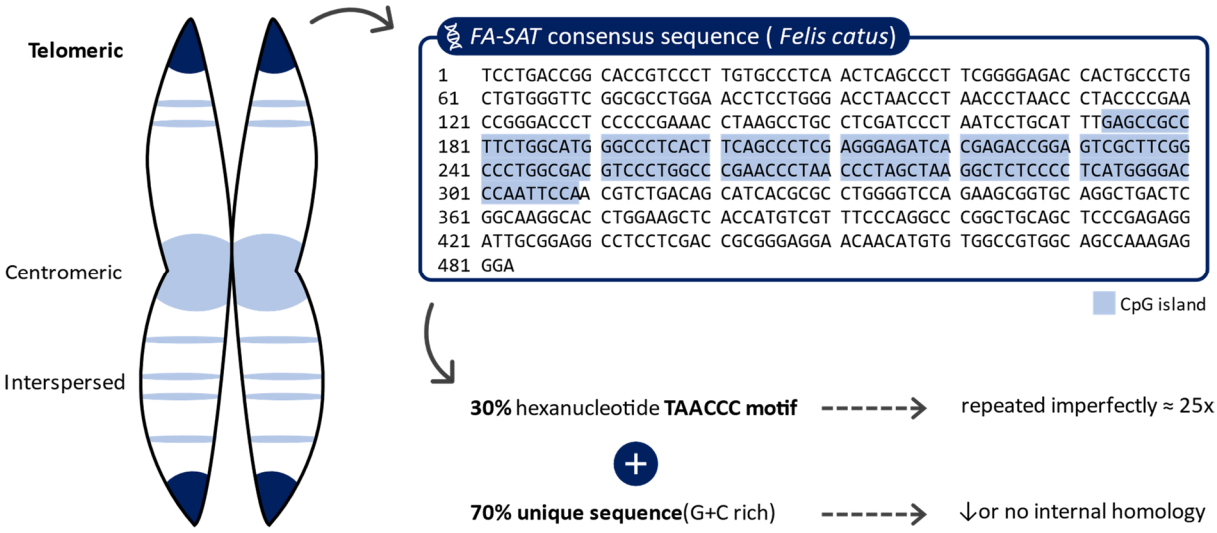

Figure I.2 - *FA-SAT* consensus sequence, monomer composition and distribution in cat chromosomes. The *FA-SAT* monomer is composed of a hexanucleotide TAACCC motif, repeated about 25 times with some variations (representing 30% of the monomer sequence) and by a unique G+C rich sequence (70% of the monomer). This satDNA is located mainly at the telomeres of the cat chromosomes, but it was also detected at the centromeres or interstitially. The CpG island reported by Chaves et al. (2017) is highlighted in blue, in the consensus sequence.

The fact that this sequence plays such a central role in the genome of the domestic cat (especially within the group of repetitive DNA sequences), has stimulated the scientific community to investigate whether *FA-SAT* could also be present in the genomes of other species and to which level of similarity/homology could be found in them.

I.2.1. *FA-SAT* is conserved and transcribed in several Bilateria species

As previously referred, satDNAs, generally, are not well conserved between distant taxa, due to the mechanisms responsible for their generation and evolution. However, some "frozen" satDNAs, as *FA-SAT*, remain preserved in the genomes, throughout the evolutionary process, showing high similarity and reflecting conserved functions of these sequences, shaped by selective constraints (Ugarkovic 2005; Biscotti et al. 2015; Chaves et al. 2017).

A few years ago, Fanning had already reported the existence of *FA-SAT*-related sequences, in different mammalian species from Carnivora, Chiroptera, Lagomorpha, Primates and Rodents orders, presenting sequence homology, but different amounts and distribution (not tandemly arranged as in the cat genome) (Fanning 1987; Fanning et al. 1988). More recently, Zou and Li also reported the existence of a *FA-SAT*-related sequence in the *Ursus thibetanus* Cot-1 DNA, presenting an interspersed distribution pattern (Zou and Li 2012).

In 2017, Chaves and colleagues analyzed and compared the *FA-SAT* sequence, organization and transcriptional activity, in the genomes of 14 Bilateria species (including

human species), from 9 different orders. They discovered that these sequences could be found in all the Bilateria species analyzed (comprising mammalian and non-mammalian species), with low intra- and inter-sequence variability and presenting conservation of the CpG island existing in the *FA-SAT* monomer (concerning its sequence and methylation status). Regarding the organization of *FA-SAT*-related sequences in the different genomes, they noticed two distinct distribution patterns: in non-Carnivora genomes, these sequences presented an interspersed pattern; in Carnivora genomes, although some copies of these sequences showed an interspersed distribution, it seemed to predominate the occurrence of the *FA-SAT* monomer amplification and, consequently, the formation of long tandem arrays in heterochromatic regions. Finally, the authors detected transcriptional activity of the *FA-SAT*-related sequences in all Bilateria species analyzed, yielding *FA-SAT* ncRNA (Chaves et al. 2017; Ferreira 2018).

Although differences in copy number and distribution of *FA-SAT*-related sequences were observed in these genomes, all these sequences show high homology (*FA-SAT* is considered the most conserved satDNA described hitherto). This, allied with its transcription in distant Bilateria species, may let us anticipate conserved functions for these satellite transcripts (Palazzo and Gregory 2014; Chaves et al. 2017; Ferreira 2018), which deserve to be explored in detail.

I.2.2. *FA-SAT* ncRNA characterization and its (un)known functions

In recent times, the study of satDNA sequences and their activity has been greatly boosted (Ugarkovic 2005; Biscotti et al. 2015). *FA-SAT,* as a highly conserved and transcriptionally active sequence (originating *FA-SAT* small and long ncRNAs), has newly emerged as a potential key player and contributor to important biological processes (Chaves et al. 2017; Ferreira et al. 2019a; Ferreira et al. 2019b). The study of the activity of its transcripts is crucial to unveil their putative roles in regulating different cellular and molecular mechanisms and to compare the functions played by them in distinct taxa or in different cells from the same species (Chaves et al. 2017; Ferreira 2018).

Ferreira et al. (2019a) performed the cellular characterization of the *FA-SAT* transcripts in a cat proliferative cell line, FCAn. They observed that *FA-SAT* ncRNA is located at the nucleus and nucleolus, but it is not observed in all the cells. Then, they determined that *FA-SAT* transcription is cell-cycle dependent, with *FA-SAT* ncRNA accumulating mainly in the late G1 and G1/S transition (but also observed in G0/early, G1 and in S phase). These data indicate a

nuclear function of *FA-SAT* ncRNA, in these phases of the cell cycle (Ferreira 2018; Ferreira et al. 2019a).

In another work, the cellular profile of the *FA-SAT* ncRNA and its possible involvement in the tumorigenesis process was assessed in several passages of a cat mammary tumor cell line, FkMTp (Borges et al. 2016; Ferreira 2018), evaluating the *FA-SAT* behavior during the *in vitro* tumor progression/evolution (Ferreira 2018). Compared with the non-tumor cat cells (Ferreira et al. 2019a), FkMTp cells showed analogous nuclear and nucleolar locations of the *FA-SAT* transcripts, but distinct distribution of these transcripts throughout the cell cycle (*FA-SAT* ncRNA signals were found in all cell cycle phases), as shown in Figure I.3. Thereby, it was possible to infer that *FA-SAT* was dysregulated in the cell cycle of the cat tumor cells (Ferreira 2018). Concerning the behavior of *FA-SAT* ncRNA in the different passages: it was possible to observe a higher variation of the amount and distribution of these transcripts in the cells of the first 2 passages (due to the initial cellular heterogeneity); its cell cycle distribution in the first 2 passages showed to be closer to the profile obtained in the FCAn cells (the tumor progression must have caused an increasing dysregulation of the *FA-SAT* expression/degradation and the cell cycle itself) (Ferreira 2018; Ferreira et al. 2019a).

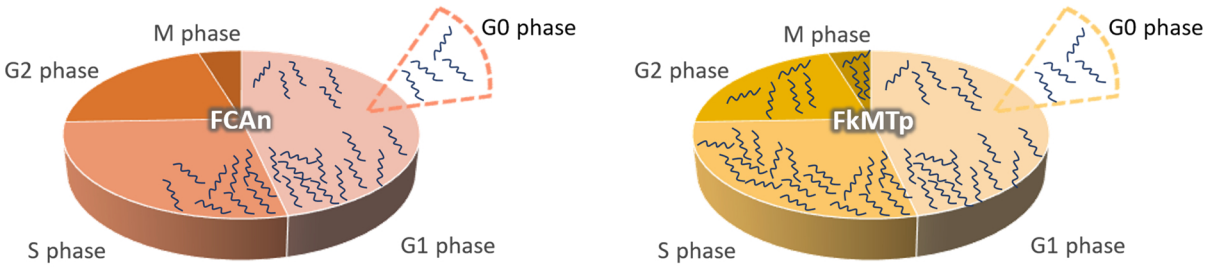

Figure I.3 - Distribution of *FA-SAT* transcripts throughout the cell cycle of the FCAn and FkMTp cell lines. In the FCAn cells, the *FA-SAT* ncRNA accumulates mainly in the late G1 and G1/S transition, but it is also observed in G0/early G1 and onset of S phases (cell-cycle dependent expression). In the FkMTp cells, *FA-SAT* ncRNA signals were found in all cell cycle phases (dysregulated expression).

Ferreira and colleagues also carried out functional assays for *FA-SAT* ncRNA, starting with the study of its putative protein interactors. PKM2 (Pyruvate Kinase Muscle Isozyme), a moonlighting protein with several cytoplasmic and nuclear functions, was identified as the main interactor of *FA-SAT* ncRNA. After that, silencing/ectopic expression experiments were done in the FCAn cell line and in human HeLa cells. They reported that: 1) when *FA-SAT* was silenced, a decrease in *PKM2* RNA and protein levels occurred (especially in the nuclear PKM2 protein); 2) the interference with *PKM2* RNA (using a siRNA) corresponded to a reduction of

the *FA-SAT* ncRNA levels. Additionally, the silencing of either *FA-SAT* or *PKM2* (with consequent disruption of this ribonucleoprotein complex) resulted in an apoptosis phenotype, reduced levels of *MYC* RNA and protein (mitogenic pathway) and increased levels of P53 protein (apoptotic pathway). The ectopic overexpression of *FA-SAT* led to the PKM2 nuclear accumulation. So, the authors pointed out that *FA-SAT* transcripts might be responsible for recruiting the PKM2 protein to the nucleus, allowing it to perform its nuclear function (Ferreira 2018; Ferreira et al. 2019a). Together, they should be involved in the regulation of the switch between cell-cycle progression and apoptosis (especially in G1/S transition), presenting a conserved function in cat and human cells (Ferreira 2018; Ferreira et al. 2019a).

The *FA-SAT* silencing in FkMTp cells, in all cell line passages, resulted in the same molecular and cellular phenotypes (i.e., apoptosis and decreased levels of *PKM2* and *MYC* RNA), supporting the existence of a conserved nuclear function of the *FA-SAT* ncRNA/PKM2 complex, in non-tumor and tumor cat cells. Even more, the authors purposed that the functionality of this ribonucleoprotein complex should be defined by the number of interactions between the *FA-SAT* ncRNA and the PKM2 protein (and not necessarily by the individual amounts of the two interactors) (Ferreira 2018; Ferreira et al. 2019a).

Finally, Ferreira and colleagues investigated the putative influence of the *FA-SAT* ncRNA/PKM2 complex state in the determination of the FkMTp sensitivity/resistance to some antimitotic drugs. In this work, 4 passages of the FkMTp cell line were used, the cell behavior/fate was followed by time-lapse microscopy (after the exposure to the antimitotic drugs) and the *FA-SAT* and *PKM2* RNA levels were measured. FkMTp cells revealed to be resistant to taxol (evading apoptosis) and to acquire sensitivity to nocodazole throughout the passages (with an increasing number of cells in apoptosis). These cells showed, during taxol treatment, maintenance or increase of the levels of *FA-SAT* ncRNA and maintenance of the *PKM2* RNA levels. During the nocodazole treatment, the *FA-SAT* ncRNA levels were maintained and the *PKM2* RNA levels decreased. As a result, the authors suggested that the number of interactions between the *FA-SAT* ncRNA and the PKM2 protein should be implicated in determining the cell sensitivity/resistance to the antimitotic drugs and, consequently, the cell fate (Ferreira 2018).

The *FA-SAT* ncRNA/PKM2 complex dynamics and some molecular phenotypes associated with it are summarized in Figure I.4.

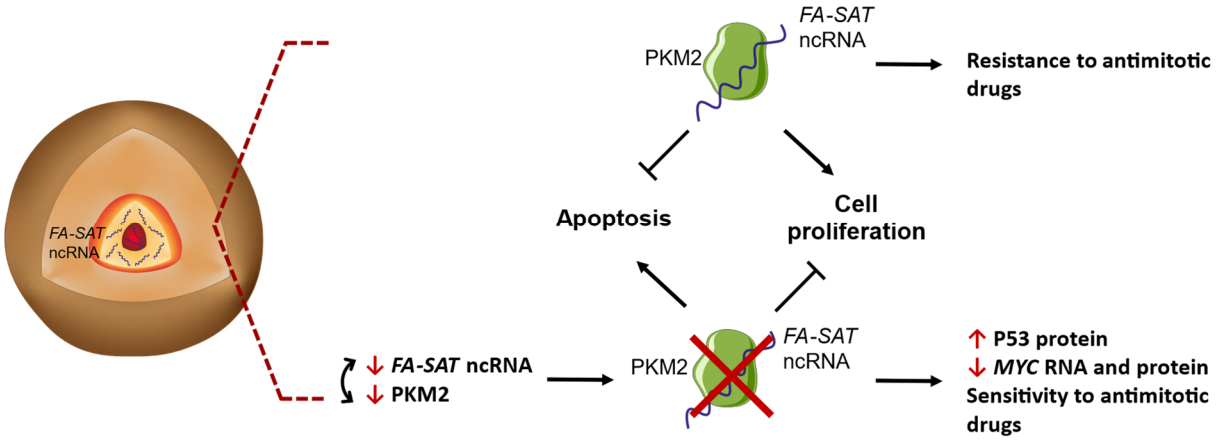

Figure I.4 - *FA-SAT* ncRNA/PKM2 interaction and different outcomes determined by its state. *FA-SAT* ncRNA interacts with PKM2 protein to perform nuclear functions, regulating the switch between cell proliferation and apoptosis and determining the cell sensitivity/resistance to some antimitotic drugs.

Due to the roles played by this complex in regulating the expression of several critical cancer genes and determining the cell fate (cell proliferation, cell death or evasion to apoptosis), it became necessary to understand how *FA-SAT* expression is controlled (Subchapter I.2.3.) and how its dysregulation can contribute to different features and hallmarks of cancer (Subchapter I.2.4).

I.2.3. (Dys)Regulation of *FA-SAT* expression

It has already been described that the alterations in the expression of some satDNAs (for example, their overexpression due to hypomethylation) can be related to the genomic instability of cancer cells and their aberrant behavior (Ting et al. 2011; Ferreira et al. 2015; Saksouk et al. 2015). The influence of *FA-SAT* ncRNA in different and important cellular pathways, that are affected by the hallmarks of cancer (Hanahan and Weinberg 2000; Hanahan and Weinberg 2011), highlights the importance of the *FA-SAT* study in cancer cells.

The *FA-SAT* DNA amplification in a cat fibrosarcoma was reported by Santos et al. (2006). They reported that, in tumor cells, this variable number of signals (number of copies) and appearance, observed by DNA-FISH, could be related to the chromosomal and mitotic instability of the cat fibrosarcoma cells. Though, the *FA-SAT* ncRNA levels were not assessed in this study (Santos et al. 2006).

Ferreira and colleagues, on the other hand, evaluated the copy number variation of the *FA-SAT*, its expression, and its physical distribution in the chromosomes of different passages of a cat mammary tumor (FkMTp) cell line and in a cat non-tumor (FCAn) cell line. In all the 4 FkMTp passages examined (by DNA-FISH), there was no evident alteration neither of the

primary locations of the *FA-SAT* nor the amplification of the *FA-SAT* DNA throughout the chromosomes (compared to the amount and distribution of this satDNA in FCAn cells, and contrasting with the data reported by Santos et al. (2006)). Nevertheless, through real-time qPCR quantification, they were able to detect the amplification of the *FA-SAT* DNA in the last 2 passages of FkMTp cells. Besides, they analyzed the *FA-SAT* ncRNA levels (small and long RNA fractions) and concluded that the overexpression of this satDNA was not associated with its amplification (the passages that presented *FA-SAT* DNA amplification did not demonstrate *FA-SAT* overexpression and vice versa) (Ferreira 2018; Ferreira et al. 2019b). The *FA-SAT* DNA copy number and its expression were also investigated in samples of fresh feline mammary tumors (FMT) and the respective disease-free tissues (DFT). In these samples, the amplification of *FA-SAT* DNA was not observed, but the *FA-SAT* small RNA is overexpressed. Consequently, the authors assumed that, in these samples, *FA-SAT* overexpression could not be explained by its amplification, agreeing with the data obtained for the FkMTp cells (Ferreira 2018; Ferreira et al. 2019b).

As mentioned before, it has been reported the existence of a CpG island in the *FA-SAT* sequence and the conservation of its methylation status in different Bilateria species, indicating that the expression of this satDNA could also be controlled by this epigenetic mechanism (Fanning 1987; Chaves et al. 2017). Thereby, the *FA-SAT* methylation profile was explored in FCAn and FkMTp cells and in cat tissue samples (FMT and the respective DFT). In the FCAn and FkMTp cell lines, most of the passages presented methylation of the *FA-SAT* DNA and there was a correspondence between the lower levels of methylation in a specific FkMTp passage and the overexpression of *FA-SAT*. The *FA-SAT* demethylation resulted in its overexpression in FCAn, both in the small and long RNAs, but in FkMTp cells only the *FA-SAT* small RNA fraction was increased. The analysis of the FMT samples enabled us to infer that the *FA-SAT* was mainly hypomethylated or presented a similar methylation profile (compared to the respective DFT sample) and, in most of the analyzed samples, no association between the *FA-SAT* methylation status and its transcription levels was found. All these data suggested that DNA methylation could be involved in the regulation of the *FA-SAT* expression, however, in cancer cells (FkMTp and FMT), additional mechanisms should contribute to its transcriptional status (Ferreira 2018; Ferreira et al. 2019b).

The information about the cellular processes responsible for the (dys)regulation of the *FA-SAT* transcription is still scarce. The deepening of this study, in non-tumor and tumor cells, will be fundamental to understand how this satDNA can contribute to different cancer cell

phenotypes, and to evaluate its putative value as a predictive and/or target molecule in cancer diagnosis and therapy.

I.2.4. *FA-SAT* DNA and ncRNA as potential cancer biomarkers

In previous subchapters, it was mentioned that *FA-SAT* ncRNA/PKM2 complex could be linked to cancer hallmarks, such as increased cell proliferation and/or evasion to apoptosis (Santos et al. 2006; Hanahan and Weinberg 2011; Ferreira 2018; Ferreira et al. 2019a). It is now necessary to disclose its association with other features of tumor cells, its involvement in the tumorigenesis process and its potential as a cancer biomarker.

In 2018, it was investigated the putative association of the *FA-SAT* DNA and its transcripts with some clinicopathological parameters in FMT and DFT samples. The *FA-SAT* DNA copy number was correlated positively with lymphatic invasion and *ERBB2* DNA levels, and negatively with the overall survival (the existence of lymphatic invasion and the amplification of the oncogene *ERBB2* lead to the poor patients' prognosis, resulting in less overall survival) (Peiró et al. 2007; Deger et al. 2015; Ferreira 2018). Besides, the *FA-SAT* long RNA levels were negatively related to the ki67 index and malignancy grade (the tumors with lower levels of these transcripts will present a worse clinical outcome). These long transcripts also showed differential expression, depending on the molecular classification of the tumor (its quantification may help in the identification and distinction of different mammary tumor subtypes). Finally, the small fraction of the *FA-SAT* ncRNA showed to be positively associated with the tumor size (in larger tumors, more *FA-SAT* ncRNA processing should occur and higher levels of small RNAs will be obtained) (Ferreira 2018).

Therefore, due to the association of the *FA-SAT* and its transcripts with several clinicopathological tumor features and different hallmarks of cancer, they present potential to be used as biomarkers for diagnosis, prognosis and choice of therapy in cancer (Ferreira 2018).

I.3. PKM2, the main interactor of *FA-SAT* ncRNA

Pyruvate kinase (PK) is a metabolic enzyme that catalyzes glycolytic reactions and can present four different isoforms (M1, M2, L and R) in mammalian species, depending on cell/tissue type (Tanaka et al. 1967; Imamura and Tanaka 1972; Nakashima et al. 1974; Amin et al. 2019).

M1 and M2 are both encoded by the *PKM* gene, being the isoform determined by a process of alternative splicing of the *PKM* pre-mRNA, as shown in Figure I.5: if the exon 9 was included and the exon 10 excluded, the PKM1 isoform will be obtained; if the opposite scenario happens, PKM2 will be produced (Noguchi et al. 1986; David et al. 2010). While PKM1 is predominantly expressed in terminally differentiated tissues, such as muscle and brain (Corcoran et al. 1976), PKM2 is mainly observed in high proliferation environments, such as stem cells, embryonic cells and cancer cells, being frequently upregulated in the last (David et al. 2010; Mazurek 2011).

PKM2 protein is firstly known for its important role in catalyzing the final step of glycolysis, enabling the conversion of phosphoenolpyruvate (PEP) and adenosine diphosphate (ADP) into pyruvate and adenosine triphosphate (ATP) (Staal and Rijksen 1991). In non-tumor cells, pyruvate is either oxidized to CO_2 to produce more ATP in the mitochondrial respiratory chain (in the presence of oxygen and functional mitochondria) or converted into lactic acid (if oxygen or mitochondria are absent) (Bose et al. 2021). In cancer cells, the presence and upregulation of PKM2 seem to contribute to the metabolic reprogramming and to the switch from the mitochondrial oxidative phosphorylation to aerobic glycolysis (regardless of oxygen state), known as Warburg effect (Warburg et al. 1927; Christofk et al. 2008). The preference for glycolysis makes cells less efficient for ATP production, but it enables the redirection of some glycolytic intermediates to the biosynthesis of macromolecules. Thus, cancer cells can attend to their needs for high proliferation rates, combining the production of energy and macromolecules, in a faster process (Vander Heiden et al. 2009; reviewed in Wong et al. 2013; reviewed in Cannino et al. 2018; Bose et al. 2021). Besides that, PKM2 presents several additional non-metabolic functions, being considered a moonlighting protein (Iqbal et al. 2014). For example, in mitochondria, it regulates the morphology of this organelle, reduction of ROS (reactive oxygen species) damage and mitochondrial-dependent apoptosis (Amin et al. 2019).

It is known that PKM2 can be found in the cells in both the tetramer and dimer forms, depending on the subcellular location of the protein and the function it is performing (Mazurek et al. 2005; Gao et al. 2012). At the cytoplasm and mitochondria, it can be observed in the tetramer and/or the dimer forms. On the other hand, at the nucleus, it is only found in the dimer state, being this conversion needed for its translocation to this cell organelle (Wong et al. 2013; Amin et al. 2019; Zhang et al. 2019). Some biomolecules/metabolites and specific posttranslational modifications (such as phosphorylation, oxidation and acetylation), induced by endogenous or exogenous stimuli, can catalyze the tetramer-to-dimer conversion and also

promote the PKM2 nuclear translocation. For example, the EGFR (epidermal growth factor receptor) stimulation triggers the ERK-mediated phosphorylation of PKM2 (at serine-37), with a cascade of events that result in the PKM2 nuclear translocation and, there, induction of *MYC* expression (Amin et al. 2019). In fact, at the nucleus, PKM2 acts as a protein kinase (Harris et al. 2012; Iqbal et al. 2014) with several targets, including histone H3, Bub3 (spindle checkpoint), Bcl-2 (apoptosis regulator), among others (Yang and Lu 2015; Dong et al. 2016; Xia et al. 2016; Liang et al. 2017), participating in the regulation of the expression of some genes (Tamada et al. 2012b; Dong et al. 2016) and being described as potentially engaged in the tumorigenesis of a variety of cancers (Zhang et al. 2013; Wang et al. 2015; Azoitei et al. 2016; Dayton et al. 2016).

Among the genes that have their expression regulated by PKM2, there are some critical cancer genes, such as *CCND1* (Cyclin-D1), *MYC, STAT3* and, in the last instance, *PKM2* itself (David et al. 2010; Yang et al. 2011; Gao et al. 2012; Yang et al. 2012a; Yang et al. 2012b). For instance, PKM2 presents the ability to interact with β-catenin to phosphorylate histone H3 at the *CCND1* and *MYC* promoter regions, resulting in the HDAC3 dissociation, acetylation of histone H3 and, finally, transcription of *Cyclin-D1 and MYC* (Yang et al. 2011; Yang et al. 2012a; Dong et al. 2016; Amin et al. 2019). Beyond that, PKM2 interrelates with the c-MYC protein in a positive feedback-loop once c-MYC acts as a transcription factor to regulate the alternative splicing of the *PKM* gene, repressing exon 9 encoding pre-mRNA sequences and promoting the expression of the PKM2 isoform (David et al. 2010).

In 2016, Xia and collaborators stated that PKM2 can act as a P53 co-repressor, inhibiting the transactivation of the *p21* gene and leading to inhibition/evasion to apoptosis. Additionally, the non-stop in the G1 phase/DNA damage checkpoint (regulated by P53) will perpetuate the DNA damage and cause genomic instability (Xia et al. 2016). In turn, PKM2 will interact and phosphorylate histone H2AX (at serine residue S139), which can be considered a sensitive marker for the occurrence of double-strand breaks (Xia et al. 2017).

Some authors support the importance of PKM2 in promoting inflammatory responses, through metabolic reprograming (Yang et al. 2014; Palsson-McDermott et al. 2015). Moreover, it was reported that this protein is implicated in the angiogenesis process observed in tumor cells, namely in the regulation of the VEGF (vascular endothelial growth factor) expression and secretion in endothelial cells (Xu et al. 2015; Azoitei et al. 2016), and in invasion and metastasis mechanisms (Liu et al. 2014). Finally, PKM2 seems to be decisive in the resistance acquisition to several chemotherapeutic drugs, with its depletion resulting in the sensitization of different

types of cancer cells to those drugs (Shi et al. 2010; Tamada et al. 2012a; Li et al. 2015; Pan et al. 2016).

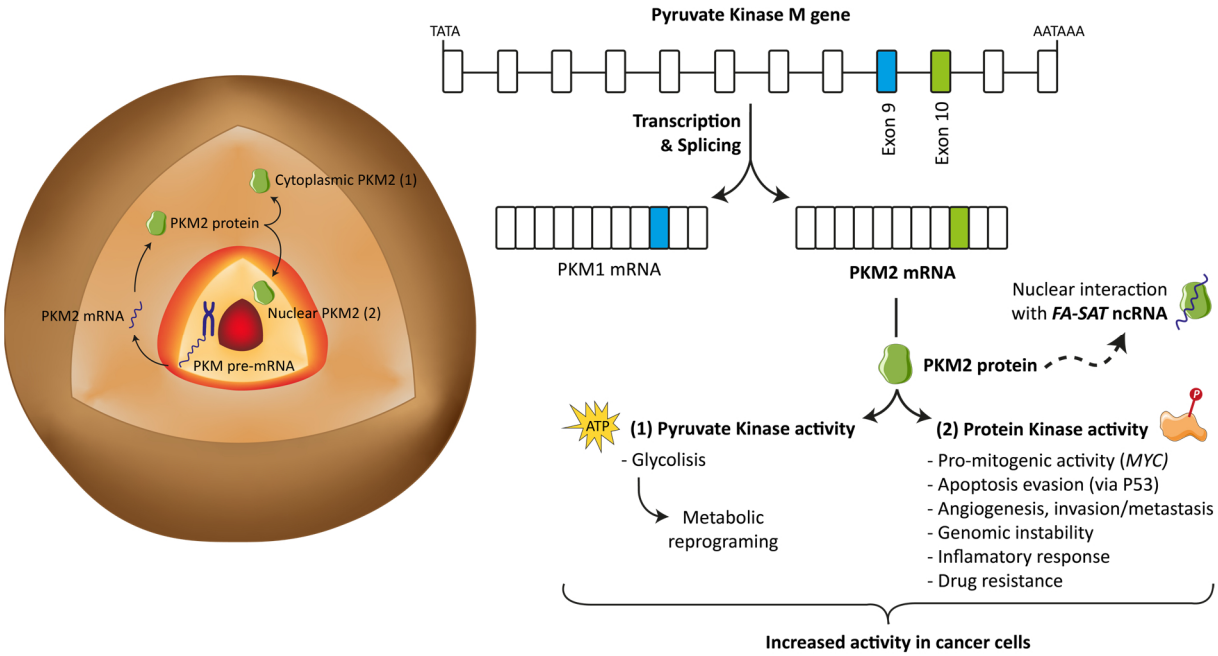

Figure I.5 - Schematic representation of the PKM2 origin, cell distribution and functions played by it. PKM2 is obtained through alternative splicing of the PKM pre-mRNA, by the inclusion of exon 10 (and exclusion of exon 9). In the cytoplasm, this protein acts as pyruvate kinase and catalyzes the last step of glycolysis. In the nucleus, it performs functions as protein kinase (and interacts with *FA-SAT* ncRNA), participating in the regulation of several processes, such as transcription, cell proliferation, apoptosis, among others. In tumor cells, PKM2 is frequently upregulated, contributing to cancer progression.

The data here described seems to agree with the *FA-SAT* ncRNA/PKM2 functions reported (Subchapter I.3.2.), once *FA-SA*T ncRNA/PKM2 depletion is associated with: downregulation of *MYC* and inhibition of cell proliferation; P53 accumulation and activation of apoptotic pathway(s); sensitization to anti-mitotic drugs (such as taxol and nocodazole) and, therefore, cell death (Ferreira 2018).

Thus, we can infer that PKM2 generally performs pro-mitogenic and/or anti-apoptotic functions (Dong et al. 2016; Snaebjornsson and Schulze 2018), contributing to the abnormal proliferation of tumor cells and to several cancer hallmarks, including apoptosis evasion, genomic instability, inflammation, angiogenesis, invasion/metastasis and dysregulation of cellular energetics. In this sense, PKM2 can be considered a cancer key player and, together with *FA-SAT* ncRNA, must be explored, in order to unveil its/their potential as cancer biomarker(s) and target(s) for cancer diagnosis and therapy.

I.4. Mitogenic and apoptotic signaling by *MYC* and *P53*

In cancer cells, malignancy is the result of the accumulation of several mutations that are acquired successively and that are perpetuated over time. In the non-tumor cells, some mechanisms guarantee their integrity, checking and repairing DNA errors (that can occur during the replication/cell division process) and restoring normality to the cell cycle or activating programmed cell death pathways (preventing errors from spreading) (Hanahan and Weinberg 2000; Hanahan and Weinberg 2011). However, under certain conditions, the cells can overcome these internal checkpoints and develop resistance to cell death, carrying with them several critical mutations and some carcinogenic potential (Evan and Vousden 2001; Igney and Krammer 2002).

There is a vast list of genes/proteins that may be involved in the regulation of the cell cycle, either in maintaining its normal progression or in verifying the integrity of the process (checkpoints) and, if necessary, activating pathways of last resort (cell death). Among them are the well-known (proto-)oncogene *MYC* and the tumor suppressor gene *P53* (Dang 1999; Chari et al. 2009; Yu et al. 2019).

MYC is considered a key player in cell life, acting as a transcription factor of numerous genes/targets and as an activator or inhibitor of various downstream pathways. Due to its functions, it can regulate cell processes, such as cell proliferation, DNA replication, metabolism, differentiation and apoptosis/cell death (Dang 1999; reviewed in Bretones et al. 2015; Yu et al. 2019). Concretely, when there is a need for cells to meet high proliferation rates and in response to mitogens/growth factors, *MYC* is upregulated through transcriptional and post-transcriptional modulation (highlighting the important role of RAS/RAF/MEK/ERK cascade in it) (Waters et al. 1991; Kerkhoff et al. 1998). Then, there is a consequent increase in the expression/activation of the MYC targets that are able to promote cell proliferation (e.g., *CCND1/2*, *CCNE1*, *CCNA*, *CDK4*, *E2F1/2*, *CDC25*, among others) and, on the other hand, downregulation/repression of target genes involved in cell-cycle arrest (e.g., cyclin-dependent kinase inhibitors, as *p21* and *p27*) (Lutz et al. 2002; Meyer and Penn 2008). On the contrary, the response to antiproliferative signals (e.g., interferon-γ or transforming growth factor β), results in *MYC* downregulation and impairment of cell proliferation (Pietenpol et al. 1990; Ramana et al. 2000; Wang et al. 2008). This scenario of *MYC* downregulation can also be verified when the cells experiment proliferation arrest and undergo terminal differentiation (Prochownik et al. 1988; Leon et al. 2009). Still, when the cells are exposed to certain stimuli such as DNA damage, glucose deprivation, hypoxia, heat shock, chemotherapeutic agents,

continuous growth arrest and others, MYC can induce or sensitize cells to apoptosis, in a P53-dependent or -independent way (determined by the cell type and the cell conditions/apoptotic trigger) (reviewed in Maddika et al. 2007; Hoffman and Liebermann 2008).

Furthermore and associated with all the functions described above, MYC is frequently dysregulated in cancer, with nearly 50% of human solid tumors and leukemia presenting alterations in its activity, related to tumorigenesis (Dang 1999; Vita and Henriksson 2006; reviewed in Bretones et al. 2015). In these cells, mutations that directly affect *MYC* or mechanisms that contribute to altering its activity (proto-oncogene to oncogene conversion) often lead to gains in its function and uncontrolled increase in cell proliferation, combined with genomic instability and evasion to cell-cycle arrest/cell death (reviewed in Lutz et al. 2002; Yu et al. 2019).

In turn, P53 has come to be called "the guardian of the genome", as it contributes to processes such as DNA repair, response to stress, senescence, cell-cycle arrest, cell death, differentiation, among others (Stiewe 2007; Chari et al. 2009; Vousden and Prives 2009). Its activity in the cell can be performed in a transcription-dependent or -independent way: at the nucleus, P53 acts as a transcription factor of several genes that are involved in the cell-cycle progression, checkpoints, apoptosis, signaling, metabolism and regulation of the expression of other genes; at the cytoplasm, its transcription-independent activities are related to the promotion of cell death, by apoptosis or autophagy (Stiewe 2007; Xu-Monette et al. 2012). In fact, some of the most significant roles associated with this protein are the cell-cycle arrest and apoptosis induction, via activation of downstream effectors (that may include BAX, PUMA and NOXA) or repression of the activity of pro-mitogenic targets (e.g., MYC) (Miyashita and Reed 1995; Oda et al. 2000; Nakano and Vousden 2001; Ho et al. 2005). This response is often triggered by DNA damages and/or cell growth control and may involve distinct signaling pathways, of which we can highlight the P53 phosphorylation via ATM/CHK2/1 and ATR/CHK1 cascades or the inhibition of the MDM2 protein by $p19^{Arf}$ (all of them resulting in the P53 stabilization and activation) (Xu and Baltimore 1996; Zhang et al. 1998; reviewed in Carr and Jones 2016).

Due to its central role in cell function and specifically in the cell division process, P53 malfunction is commonly implicated in cancer (Soussi and Wiman 2007): it is one of the most frequently altered gene/protein in human cancer cells (Chari et al. 2009), with approximately 50% of human tumors carrying *P53* mutations (Hollstein et al. 1994; Soussi and Wiman 2007) or presenting other mechanisms that can compromise wild-type P53 function (Wiman 2006;

Kruse and Gu 2009). In both cases, the loss of the function of this protein (Ang et al. 2006) has consequences in terms of resistance to cell death, increased cell proliferation and genomic instability (Chari et al. 2009) and contributes to tumor initiation, maintenance, progression and metastasis (Martins et al. 2006; Ventura et al. 2007; Xue et al. 2007).

Recently, it was stated that some ncRNAs can contribute also to the control of cell-cycle progression and cell-cycle arrest/cell death (Jovanovic and Hengartner 2006; Kitagawa et al. 2013; Do and Kim 2018). Considering the central role that MYC and P53 play in the mitogenic and apoptotic pathways, it is quite acceptable to assume that these key players can interrelate with these ncRNAs, regulating and being regulated by them (He et al. 2007; Hermeking 2007; Hart et al. 2014; Stasevich et al. 2021). For that reason, the interaction/association between *FA-SAT* ncRNA and MYC/P53 deserves to be explored, in order to understand the contribution of this satDNA and its transcripts to the regulation of the cell cycle in humans.

I.5. Aims of the work

In recent times, satDNA sequences and their transcripts have emerged as key players in different cellular and molecular contexts, being also associated with several diseases, such as cancer. *FA-SAT*, the major satDNA of the domestic cat genome, presents conservation of its sequence and transcription between distant species, suggesting the existence of important and conserved functions associated with it, in these taxa. In cat non-tumor and tumor proliferative cells, it was reported the interaction of the *FA-SAT* ncRNA with the PKM2 protein (forming a ribonucleoprotein complex), to perform nuclear function(s) in defining the cell-cycle progression. Furthermore, the variation of the *FA-SAT* expression levels (and consequently of the number of interactions between this satncRNA and PKM2 protein), was pointed out as being potentially correlated with some cancer hallmarks and features of the tumor cells.

The first approach to the characterization of the *FA-SAT* ncRNA in the cellular context was performed, mainly, in cat proliferative cells, once in this species there is a higher amount of these transcripts (facilitating their description and tracking in the cells) (Ferreira 2018). However, since the *FA-SAT* sequence and transcription remain preserved also in the human species and the *FA-SAT* ncRNA function is conserved in human and cat cells, we intended to focus on the study of its transcripts in human cancer cells. Even more, considering that the P53 accumulation was associated with the apoptosis outcome promoted by the *FA-SAT* ncRNA/PKM2 depletion, with this work we expected to unravel the implication of it in this

phenotype. Then, we could supply additional data about the mechanism(s) underlying the shift between cell proliferation and apoptosis of the *FA-SAT* ncRNA/PKM2 disruption in human species. So, in this work we aimed to:

- Characterize and compare the *FA-SAT* ncRNA cellular profile in different human cell lines;
- Evaluate the influence of the P53 status on determining the *FA-SAT* knockdown outcomes;
- Unclose the mechanism(s) by which the *FA-SAT* ncRNA/PKM2 complex modulates the switch between cell proliferation and apoptosis.

In Chapter I, a review was elaborated, providing an insight into the state of the art of: satDNA sequences and their transcripts; *FA-SAT* DNA and ncRNA characterization; main interactors/targets of *FA-SAT* ncRNA and roles in cell function and disease.

In Chapter II, the *FA-SAT* transcriptional profile was traced and compared in distinct human cell lines (HeLa, A549 and H1299), concerning its location, appearance and amount in these cells. Also, *FA-SAT* ncRNA was identified and located in the cell (spatial arrangement) and the cell cycle (temporal distribution) of human HeLa cells. This characterization was essential to better understand how can the differences in the *FA-SAT* expression contribute to different outcomes and to support the functional assays presented in Chapter III.

Subsequently, the cellular and molecular outcomes resulting from the *FA-SAT* knockdown were examined in P53 wild-type and P53 null human cell lines, in Chapter III. With this approach, we tried, firstly, to reveal the impact of the P53 status (presence or absence of this protein in the cells) on the *FA-SAT* depletion phenotypes. Moreover, the way by which the *FA-SAT* ncRNA/PKM2 complex regulates the mitogenic and apoptotic pathways and how it interferes with their key players (MYC and P53, respectively) was explored.

Lastly, in Chapter IV, all the data obtained were summarized, integrated and discussed, to present the main conclusions of this work. The perspectives of future work were also pointed out.

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CHAPTER II

Transcriptional characterization of *FA-SAT* ncRNA in human cancer cells

Research article:

Lucas D, Ferreira D, Adega F, Chaves R. Disclosing the *FA-SAT* ncRNA profile in human cancer cells: can its (dys)regulation be associated with some cancer features? *In preparation*.

II.1. Abstract

The advent of the study of non-coding RNAs enabled satellite DNA sequences and their transcripts to emerge as important key players in cell function and disease. *FA-SAT* is the major satellite DNA of the domestic cat and it is highly conserved and transcribed in phylogenetically distant species, including humans. Moreover, its ncRNA plays nuclear functions in regulating the cell-cycle progression, in cat and human cells. Its transcriptional characterization in cat cells showed that *FA-SAT* ncRNA is located at the nucleus and nucleolus, presenting a cell-cycle dependent distribution in non-tumor cells (detected in G0, G1 and S phases). However, since this profile was only defined in cat species hitherto, in this work we combined molecular (RT-qPCR) and cellular (RNA-FISH/IF) approaches to characterize *FA-SAT* ncRNA in several human cell lines. An initial analysis revealed a similar nuclear location of the ncRNA in all of them, but distinct appearance (clustered or scattered) and amount, depending on the cell type. A detailed examination of its profile uncovered an additional and more specific cellular location, with these transcripts being observed also at the nucleolus and its periphery in the HeLa cell line. In the same cells, this ncRNA was detected throughout the entire cell cycle, with major enrichment in the initial phases of it (late G1 and G1/S transition). These data are crucial to better understand how the differences in the *FA-SAT* expression can be associated with different outcomes and important cellular functions in human cells.

II.2. Introduction

Over the last years, it has been growing the interest in studying the part of the transcriptomes that is not involved in the protein coding, commonly named non-coding RNAs (ncRNAs). In this sense, it was found that satellite DNA (satDNA), a class of highly repetitive tandem sequences, is transcribed into ncRNAs, contributing to the regulation of important biological/cell processes and being related to some diseases, such as cancer (reviewed in Ferreira et al. 2015; Louzada et al. 2020).

FA-SAT was originally discovered in the domestic cat *(Felis catus*) genome, as the major satDNA sequence of this species (Fanning 1987). Nevertheless, the existence of *FA-SAT*-related sequences had been also described in different mammalian and non-mammalian genomes, with high sequence homology, but distinct distribution and amount (Fanning 1987; Fanning et al. 1988; Chaves et al. 2017). Recently, Chaves and colleagues reported the transcription of this satDNA in several Bilateria species, including humans, yielding *FA-SAT* ncRNA (Chaves et al. 2017).

The characterization of *FA-SAT* ncRNA in different cat cell lines revealed its localization at the nucleus and nucleolus of non-tumor and tumor cells (Ferreira 2018; Ferreira et al. 2019). In the first ones, the *FA-SAT* transcripts were only found in G0, G1 and S phases (with higher accumulation in the late G1 and G1/S transition), being defined as cell-cycle dependent (Ferreira et al. 2019). In contrast, in the tumor cells, its distribution was dysregulated and it was detected in all the phases of the cell cycle (Ferreira 2018). Furthermore, important cellular functions were described for *FA-SAT* ncRNA in cat and human proliferative cells: it interacts with the PKM2 (Pyruvate Kinase Muscle Isozyme) protein regulating the shift from cell proliferation to apoptosis (Ferreira et al. 2019).

Although the high conservation of *FA-SAT* in humans is reported and its transcripts are known to play roles in cell-cycle regulation of human HeLa cells, the *FA-SAT* ncRNA profile was only accessed in cat species, until now. So, in this work, we combined molecular (RT-qPCR) and cell biology (RNA-FISH and IF) techniques to describe the *FA-SAT* ncRNA behavior in different human cancer cells (HeLa, A549 and H1299). Initially, we compared its location, appearance and amount in the distinct cell lines and, then, we performed a detailed characterization of its spatial and temporal distribution in HeLa cells. The full characterization of its profile is essential to better understand how can this satDNA and the differences in its expression contribute to different outcomes and to associate it with important cellular functions.

II.3. Material and Methods

Cell culture

The HeLa cell line (human cervical tumor; ATCC® CRM-CCL-2) was grown in Advanced DMEM medium, supplemented with 10% FBS (Fetal Bovine Serum), 13% AmnioMax C-100 Basal Medium, 2% AminoMax C-100 supplement, 1% of antibiotic mixture (50 µg/ml penicillin, 50 µg/ml streptomycin and 100 µg/ml neomycin) and 200 mM L-Glutamine. The A549 (human lung tumor; ATCC® CCL-185) cell line was grown in F12K medium, supplemented with 10% FBS, 1% of antibiotic mixture (50 µg/ml penicillin, 50 µg/ml streptomycin and 100 µg/ml neomycin) and 200 mM L-Glutamine. The H1299 (human lung tumor; ATCC® CRL-5803) cell line was grown in RPMI medium, supplemented with 15% FBS, 1% of antibiotic mixture (50 µg/ml penicillin, 50 µg/ml streptomycin and 100 µg/ml neomycin) and 200 mM L-Glutamine. Routine procedures were used in cell culture and all the reagents were from Gibco - Thermo Fisher Scientific.

RNA extraction/isolation

The mirVana Isolation Kit (Ambion - Thermo Fisher Scientific) was used, following the manufacturer's recommendations, to isolate total RNA from HeLa, A549 and H1299 cells. Then, the TURBO DNA-free™ Kit (Ambion - Thermo Fisher Scientific) was applied for the RNA purification and, lastly, its quantification was performed using the NanoDrop 1000 (Thermo Fisher Scientific).

Real-time RT-qPCR

The Verso 1-Step RT-qPCR kit, SYBR Green, ROX (Thermo Scientific) was used, following the manufacturer instructions, to access the *FA-SAT* expression in the HeLa, A549 and H1299 cells. For that purpose, a standard curve was firstly obtained (R^2=0.99; efficiency=98.23%), as described in Chaves et al. (2017), and then the CT values of each reaction were interpolated in it. The quantification was achieved using 100 ng of RNA (of each cell line), a pair of *FA-SAT*-specific primers (Fw - AGCTAAGGCTCTCCCCTCATG; Rev - TCAGCCTGCACCGCTTCT) and following steps: 50ºC (15 min); 95ºC (15 min); 40 cycles of 95ºC (15 sec), 59ºC (45 sec) and 72ºC (1 min). Negative controls (without RNA) and replicates were included to guarantee the accuracy of the results and a melt curve was obtained to evaluate the primers' specificity. The experiments were performed resorting to a StepOne real-time PCR system (Applied Biosystems - Thermo Fisher Scientific). The StepOne software

(version 2.2.2, Applied Biosystems - Thermo Fisher Scientific) was used to analyze the data obtained.

RNA-FISH and RNA-FISH/IF

The RNA-FISH protocol was applied to HeLa, A549 and H1299 cells, in order to access and compare the *FA-SAT* transcriptional profile in them. For this purpose, the different cell lines were grown at a concentration of 100,000 cells/mL on microscope slides (Superfrost Excell, Thermo Scientific™), at 37 °C overnight. After that, the cells were washed in 1× PBS and fixed with 2% (m/v) paraformaldehyde in PBS, at RT for 20 min. Their permeabilization was achieved using 4% (v/v) Triton X-100 in PBS, supplemented with 100 µg/mL digitonin and 2 mM of Ribonucleoside Vanadyl Complex (RVC, Sigma-Aldrich - Merck Millipore), at RT for 15-20 min, and after that, their dehydration was done in ethanol baths with progressively increasing concentrations (70%, 90% and 100%). Then, the hybridization was made with a *FA-SAT* probe, obtained through PCR amplification of the *FA-SAT* cloned sequence and labeled with biotin-16-dUTP (Sigma-Aldrich - Merck Millipore), at 37 ºC overnight. After that, a series of baths (2× SSC; 0.1× SSC; 0.1× SSC; 2× SSC) was conducted at 42 ºC to guarantee a proper stringency level. The Alexa Fluor™ 555 Tyramide SuperBoost™ kit (streptavidin-conjugated, Invitrogen - Thermo Fisher Scientific) was applied to increase the sensitivity of detection of the *FA-SAT* probe and amplify its signal, following the manufacturer recommendations. Finally, the cells were mounted and counterstained using Vectashield (VectorLaboratories) mounting medium with DAPI.

To discard DNA off-targets, a similar RNA-FISH assay (with the same *FA-SAT* probe and experiment conditions) was performed, adding a RNase A (Sigma-Aldrich - Merck Millipore) treatment. Thus, after the permeabilization step, the cells were incubated with 0.1 mg/mL RNase A, for 1 h at 37 °C.

The RNA-FISH and IF methodologies were also combined to perform the characterization of the *FA-SAT* ncRNA cellular distribution in HeLa cells. The cells permeabilization was accomplished with 5% (v/v) Tween-20 in PBS (for the PCNA antibody) or 4% (v/v) Triton X-100 with 100 µg/mL digitonin in PBS (for the other antibodies), both supplemented with 2 mM of Ribonucleoside Vanadyl Complex (RVC, Sigma-Aldrich - Merck Millipore), for 15-20 min. After the blocking step, two incubations were carried on, firstly with the primary antibody (in 5% FBS) and then with the secondary antibody (in HRP-conjugated streptavidin), both for 1 h. The remaining steps and conditions were performed in the same way as described previously.

Primary antibodies: anti-fibrillarin monoclonal antibody 38F3 (1:100, mouse, Invitrogen - Thermo Fisher Scientific MA3-16771); anti-PCNA monoclonal antibody PC10 (1:100, mouse, Calbiochem – Merck Millipore NA03); anti-cyclin D1 monoclonal antibody (1:50, mouse, Merck Millipore 05-815); anti-cyclin A polyclonal antibody (1:75, rabbit, Merck Millipore 06-138); anti-cdc25 monoclonal antibody TC-15 (1:100, mouse, Merck Millipore 05-507SP); anti-phospho-histone H3 (Ser10) polyclonal antibody (1:200, rabbit, Merck Millipore 06-570).

Secondary antibodies: anti-mouse polyclonal antibody FITC conjugate (1:200, Zymed - Thermo Fisher Scientific 81-6511); anti-rabbit polyclonal antibody FITC conjugate (1:200, Sigma-Aldrich - Merck Millipore MAP132F).

Microscopy and Image analysis

The RNA-FISH and RNA-FISH/IF images were captured resorting to confocal microscopy, with a Zeiss Axio Imager Z1 microscope/LSM 510 META and the respective LSM 510 software (version 4.0 SP2). Three lasers (argon 488nm, helium-neon 543nm and diode 405nm) were used and the same microscope settings were applied to normalize the results: argon laser set at 12.9% (pinhole of 96mm/1.02 airy units), helium-neon laser at 50.8% (pinhole of 102 mm/0.98 airy units) and diode laser at 9.9% (pinhole of 112mm); 63x objective; thickness of the Z sections of 1 µm; scan speed of 4.

The captured images were then analyzed and treated with specific software: AutoQuant X3 (Media Cybernetics) for deconvolution and analysis of signals colocalization; ImageJ 1.53c (National Institutes of Health) to generate a representative TIFF image, with merged color channels and a 2D projection of the different stacks of Z-axis; Image Pro 10.0.5 (Media Cybernetics) to create 3D Iso-Surfaces (enabling to count and measure volumes of the fluorescent signals) and Ortho slices (images from different slices of the XZ and YZ orthogonal planes).

The Adobe Photoshop CC 201 software was used to optimize all the presented images (at whole image) for color, brightness and contrast.

Statistics

The one-way ANOVA test was used to compare more than two independent samples and to determine the statistical significance. The values are presented as mean ± standard deviation (SD): non-significant or ns ($p > 0.05$); * ($p \leq 0.05$); ** ($p \leq 0.01$); *** ($p \leq 0.001$); **** ($p \leq 0.0001$).

II.4. Results

Recently, the *FA-SAT* ncRNA profile was traced in different cat mammary cell lines, exhibiting similar nuclear location in them, but distinct dispersion throughout the cell cycle when comparing non-tumor and tumor cells (Ferreira 2018; Ferreira et al. 2019). Moreover, it was proven that *FA-SAT* is present, with high similarity, in the human genome (Chaves et al. 2017) and, in this species, it is also transcribed. However, until now, its transcriptional profile was only accessed in cat species. So, in this work, we developed a series of experiments to describe and compare the *FA-SAT* ncRNA behavior in different human cancer cells, regarding its location, organization and amount in the distinct cell lines and its cell-cycle distribution in HeLa cells.

FA-SAT ncRNA presents similar nuclear location, but distinct appearance and amount, in the different human cell lines

To trace the ncRNA profile(s) in the HeLa, A549 and H1299 human cells, we combined molecular and cell biology techniques, as schematized in Figure II.1a. By real-time RT-qPCR, it was possible to obtain precise and accurate data about the *FA-SAT* expression. This analysis enabled us to detect differences in the *FA-SAT* ncRNA levels across the three cell lines under study (Figure II.1b and the Supplementary Table II.1). HeLa cells showed to be the cell line with less *FA-SAT* transcripts and H1299 the one with higher levels.

The RNA-FISH methodology, on the other hand, enabled us to perform a single-cell analysis of these transcripts. For the first time, *FA-SAT* ncRNA was observed in human cells, revealing the preservation of its nuclear location (previously reported in cat cells) (Figure II.1c). However, the appearance of these transcripts was variable, predominating the clustered arrangement in HeLa and A549 nuclei and the scattered fashion in the H1299 nuclei. A similar experiment with an additional RNase A treatment step allowed us to discard DNA off-targets, once the fluorescent signals from the *FA-SAT* probe were completely abolished in the RNase-treated cells (Supplementary Figure II.1). Lastly, the confocal microscopy images were analyzed by specific software, to measure the volume of the fluorescent signals. These data revealed significant differences in the amount of this ncRNA per cell, among these three cell lines, with HeLa cells showing to be the cell line with lower *FA-SAT* transcripts volume and H1299 the cell line with higher volume (Figure II.1d and Supplementary Table II.2). Noteworthy, the data obtained by the single-cell analysis is in accordance with the quantification of *FA-SAT* ncRNA by real-time RT-qPCR.

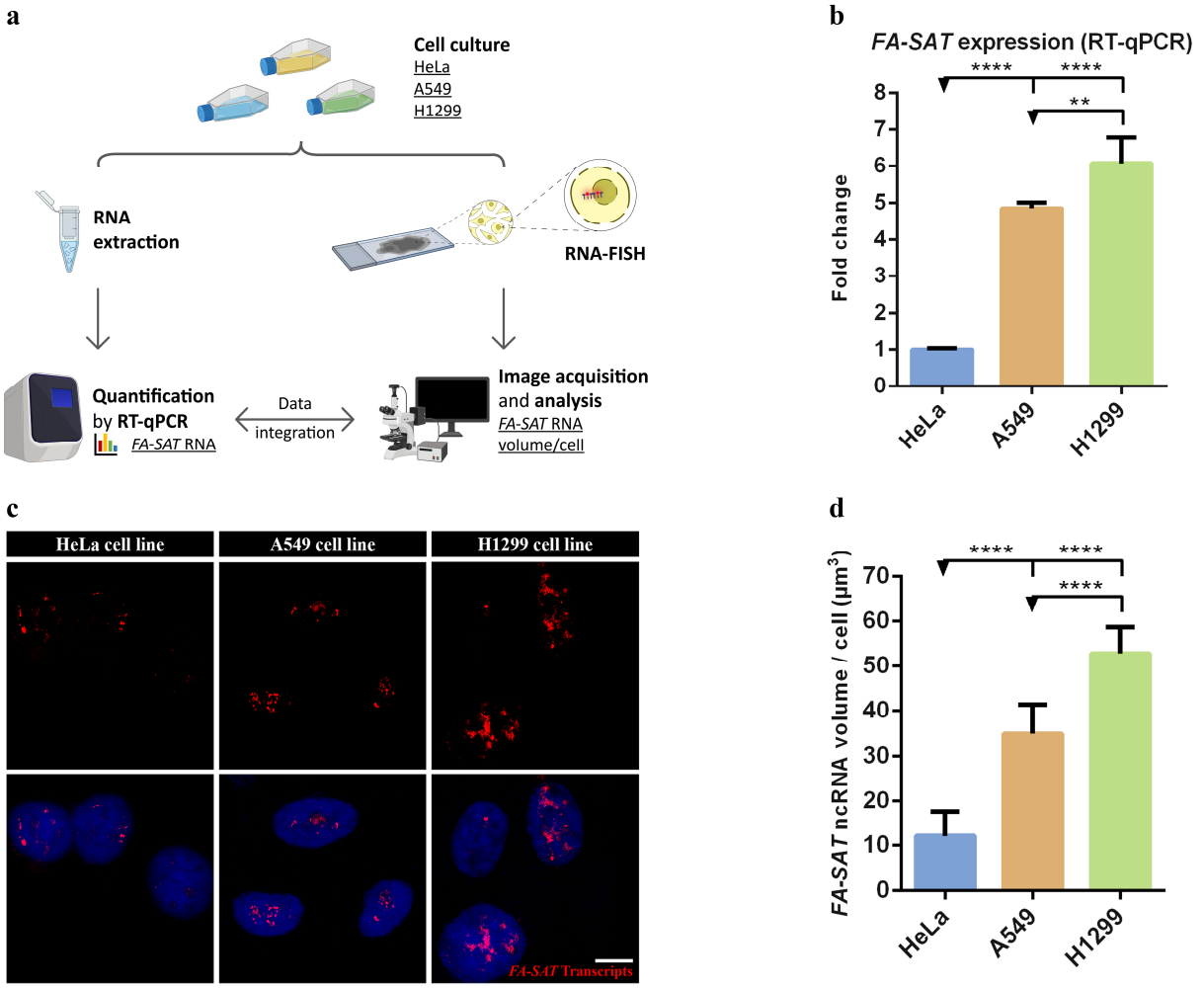

Figure II.1 - Comparison of the *FA-SAT* ncRNA profile in HeLa, A549 and H1299 human cells, regarding its location, appearance and amount. (a) Schematic representation of the *FA-SAT* ncRNA study in the different human cell lines, including molecular and cellular approaches. **(b)** Analysis, by RT-qPCR, of the *FA-SAT* expression (fold change) in HeLa, A549 and H1299 cells. **(c)** Location and organization of *FA-SAT* ncRNA in the nucleus of HeLa, A549 and H1299 cells, by RNA-FISH (red). The DNA was stained with DAPI (blue) in all the presented cells. Scale bar represents 10 µm. **(d)** Graphical representation of the *FA-SAT* ncRNA volume/cell (µm3) in the three cell lines under study, obtained by measuring the correspondent RNA-FISH signals. A minimum of 10 cells was analyzed. The statistical significance was determined by one-way ANOVA. Values are presented as mean ± SD: ** ($p \leq 0.01$); **** ($p \leq 0.0001$).

FA-SAT ncRNA is also present at the nucleolus of HeLa cells and it is detected in all the phases of the cell cycle

In the second part of our study, RNA-FISH and immunofluorescence protocols were combined to perform a more complete characterization of the *FA-SAT* transcriptional profile in a specific human cell line. As shown above, its transcripts were found at the nucleus of HeLa cells and, in this part of the work, we looked for adding information about the spatial distribution of the *FA-SAT* ncRNA in this cell organelle. Thus, examining several processed microscopy images (including representative Z projections and 3D ortho-slices/iso-surfaces), we attested the existence of co-localization between the signals from the *FA-SAT* probe and the

anti-fibrillarin antibody and proved the presence of these transcripts also at the nucleolus (and its periphery) of HeLa cells (Figure II.2a).

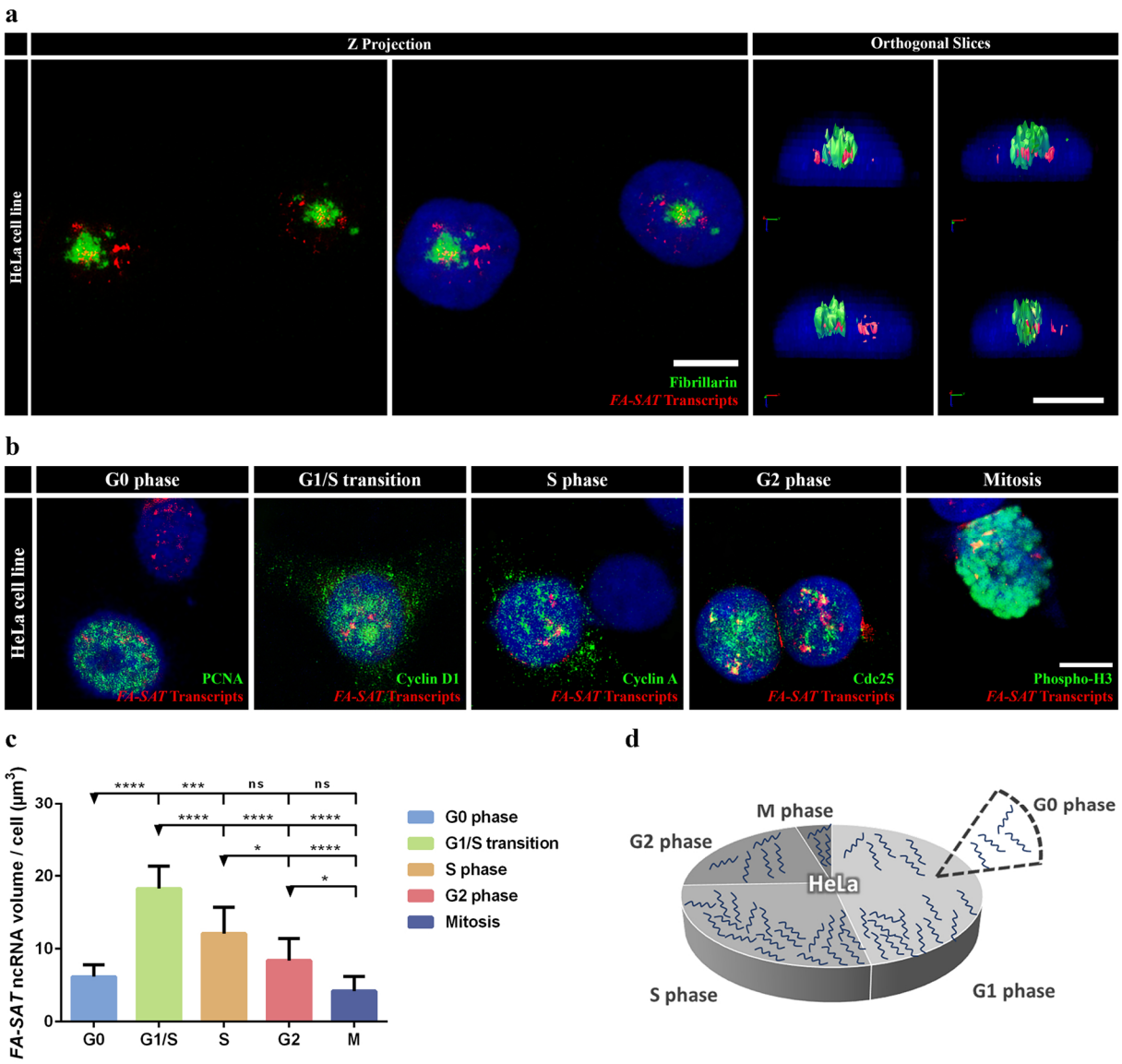

Figure II.2 - Detailed characterization of the *FA-SAT* ncRNA profile in human cells: spatial and temporal distribution in HeLa cell line. (a) Localization of *FA-SAT* ncRNA (RNA-FISH; red) at the nucleolus of HeLa cells (Fibrillarin detection by immunofluorescence; green). The 3D analysis, in the same cells, is shown with orthogonal slices and with iso-surfaces. **(b)** Cell cycle distribution of *FA-SAT* ncRNA, by RNA-FISH (red), and immunofluorescence (green) for detection of cell cycle-specific antibodies: PCNA-negative cells (G0 phase); Cyclin D1-positive cells (G1/S transition); Cyclin A-positive cells (S phase); Cdc25-positive cells (G2 phase); Phospho-histone H3-positive cells (Mitosis). The DNA was stained with DAPI (blue) in all the presented cells. Scale bar represents 10 µm. **(c)** Graphical analysis of the *FA-SAT* ncRNA volume/cell (µm^3) in each cell cycle phase. A minimum of 10 cells was analyzed. The statistical significance was determined by one-way ANOVA. Values are presented as mean ± SD: non-significant or ns ($p > 0.05$); * ($p \leq 0.05$); *** ($p \leq 0.001$); **** ($p \leq 0.0001$). **(d)** Schematic representation of *FA-SAT* ncRNA distribution in the different phases of the cell cycle.

Also, since distinct possible cell cycle arrangements had been described for *FA-SAT* ncRNA hitherto (Ferreira et al. 2019), we decide to determine which pattern is observed in HeLa cells. Using specific antibodies or markers (absence of PCNA for identification of G0 phase; presence of cyclin D1 for G1/S transition; cyclin A for S phase; cdc25 for G2 phase; phosphorylated histone H3 for mitosis), we detected *FA-SAT* transcripts in all the phases of their cell cycle (Figure II.2b). Nevertheless, analyzing the variation of the ncRNA volume/cell along the cell cycle, we observed an accumulation of it mainly in the late G1 and G1/S transition (seen in Figure II.2c and Supplementary Table II.3; schematically summarized in Figure II.2d).

II.5. Discussion

In the last years, it was proven that *FA-SAT,* the major satDNA of the domestic cat (Fanning 1987), is highly conserved and transcribed in several species, including humans (Chaves et al. 2017). Its transcriptional profile was characterized in different cat cell lines (similar nuclear location; distinct cell cycle distribution between non-tumor and tumor cells) (Ferreira 2018) and these properties were associated with the functions played by it in the cellular context: *FA-SAT* ncRNA interacts with the PKM2 protein at the nucleus of cat cells, regulating the cell-cycle progression. Furthermore, it was stated that these functions are preserved in human HeLa cells (Ferreira et al. 2019), but, until now, the profile of this ncRNA was not traced in this species. Thus, in this work, we combined molecular and cell biology techniques to characterize the transcriptional profile of this satDNA in human cell lines.

For the first time, *FA-SAT* transcripts were seen in human cells (RNA-FISH), consistently located at their nucleus, but presenting distinct arrangements in this cell organelle: the clustered organization predominated in the HeLa and A549 nuclei; the scattered fashion prevailed in the H1299 nuclei. As mentioned before, the nuclear location of this ncRNA had already been reported by Ferreira and colleagues for cat cells, and the diverse spatial arrangements of it had been described (Ferreira et al. 2019). In this sense, we could confirm the evolutionary conservation of these features of the *FA-SAT* transcriptional profile in these phylogenetically distant species. Moreover, several authors had discovered that other satncRNAs are also present at the nucleus of different cells, being able to perform regulatory functions there, through the interaction with some targets: Bury et al. (2020) demonstrated these attributes for the human α satellite (*αSat*) transcripts; Vautrot et al. (2015) for the human satellite III (*SatIII*) RNA.

The combination of the technologies used in the *FA-SAT* ncRNA quantification enabled us to complement the precision of the molecular methods, with the single-cell analysis performed by cell biology techniques. The general conclusions obtained were coincident, once significant differences in the *FA-SAT* expression were found among all the cell lines under study, in both approaches. Even so, the data obtained through RT-qPCR analysis revealed that the variations were more accentuated between cells from distinct tissues (Hela vs. A549 and HeLa vs. H1299) than between different cells from the same human tissue (A549 vs. H1299). In fact, it is known that the satncRNAs levels can vary or be altered, for example, depending on the cell type/line (Bury et al. 2020), cell conditions as stress (Jolly et al. 2003) and still in cancer (Ting et al. 2011).

In the second part of the work, we characterized the ncRNA profile in the HeLa cell line with more detail, both regarding its spatial and temporal distribution in the cells. Ferreira et al. (2019) had reported that it was possible to observe *FA-SAT* transcripts in a particular subnuclear location, namely, in the nucleolus of cat cells. In this sense, we decided to investigate whether the same was verified in this human cell line. Combining RNA-FISH and IF techniques, it was possible to detect this ncRNA at the nucleolus (and its periphery) of HeLa cells. Other satDNAs, such as *αSat,* had also their transcripts located at the nucleolus of the cells, being this feature associated with the kinetochore assembly during mitosis (Wong et al. 2007). Even more, transcripts originating from the DNA interspersed repeat Alu (SINE) were found accumulating in this subnuclear compartment of human cells, interacting there with some proteins and contributing to the preservation of the nucleolar structure and function (Caudron-Herger et al. 2015).

Finally, evaluating the *FA-SAT* expression along the division process of HeLa cells, we detected this ncRNA in all the phases of the cell cycle, similarly to what was reported by Ferreira (2018) for a cat tumor cell line. For that reason, we suggest that the expression of this satDNA is probably dysregulated in cancer cells in humans, as it happens in the cat. However, the analysis of the *FA-SAT* ncRNA volume/cell along the cell cycle shows its enrichment mainly in the late G1 and G1/S transition, emphasizing the idea that its expression may be, in fact, cell-cycle dependent. This type of regulation in the cell cycle is not exclusive of *FA-SAT* ncRNA. Previously, Lu and Gilbert (2007) demonstrated that the gamma satellite (*γSat*) transcription in mammals implied cell commitment to proliferation (Cdk dependent), with these RNAs being expressed differentially throughout the cell cycle. Also, Bury and colleagues

detected fluctuations in the *αSat* RNA amount and changes in its cellular location, both depending on the cell cycle (Bury et al. 2020).

The integration of all the data allow us to draw congruent conclusions about the *FA-SAT* transcriptional profile in human cancer cells: these transcripts presented nuclear location in all the cell lines under study (and they were also located specifically at the nucleolus of HeLa cells); they can show distinct arrangements in the nuclei (clustered and/or scattered); their amount varies among the different cell types; the *FA-SAT* ncRNA volume/cell changes over the cell cycle in HeLa cells (appearing in all its phases). Its spatial and temporal distribution in human cells and the conservation of its transcriptional profile in cat and human species corroborates the existence of nuclear functions associated with it, in these phases of the cell cycle (already described by Ferreira et al. (2019)).

This whole analysis in humans was important to establish a comparison with the results obtained in the similar studies in cat species, to complement the data existing for HeLa cells and to support the functional assays that were later performed using the A549 (P53 wild-type) and H1299 (P53 null) cell lines (described in Chapter III). Hereafter, a similar characterization should be performed in human non-tumor cells, to compare with the data here reported, so that we can infer about the state of (dys)regulation of the *FA-SAT* expression in cancer vs. normal cells and uncover its contribution to different cellular and molecular outcomes in humans.

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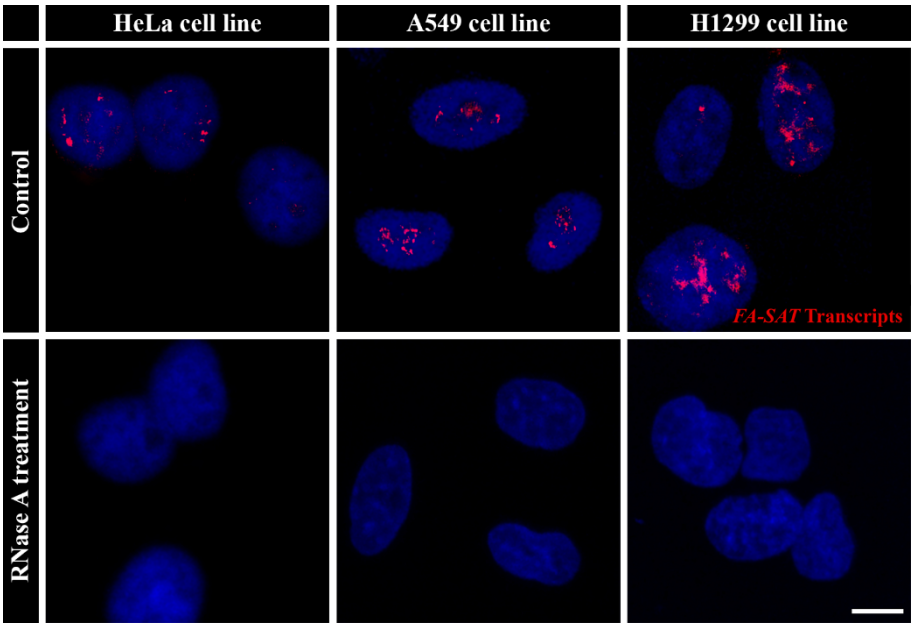
Supplementary information

Supplementary figures



Supplementary Figure II.1 - *FA-SAT* ncRNA-FISH detection in different human cell lines exposed to a RNase A treatment and in the respective control samples. *FA-SAT* ncRNA (red) observation, by RNA-FISH technique, in HeLa, A549 and H1299 cells. The comparison between the results obtained for the control cells and the respective RNase treated samples (with total absence of RNA-FISH signal in the last ones) enabled us to exclude the possibility of DNA off-targets in this experiment. The DNA was stained with DAPI (blue) in all the presented cells. Scale bar represents 10 µm.

Supplementary tables

Supplementary Table II.1 - Analysis, by RT-qPCR, of the *FA-SAT* expression (fold change) in HeLa, A549 and H1299 cells. Values are presented as mean ± SD.

		FA-SAT expression (fold change)
HeLa	RT-qPCR	1.00 (± 0.05)
A549		4.85 (± 0.19)
H1299		6.07 (± 0.86)

Supplementary Table II.2 - Analysis, by RNA-FISH, of the *FA-SAT* ncRNA volume/cell (µm3) in HeLa, A549 and H1299 cells. The RNA-FISH images were obtained through confocal microscopy and specific software was used for measuring the volumes of the fluorescent signals (per cell), in each cell line. A minimum of 10 cells was analyzed. Values are presented as mean ± SD.

		FA-SAT ncRNA volume/cell (µm3)
HeLa	RNA-FISH (signals measurement)	12.03 (± 5.49)
A549		34.87 (± 6.52)
H1299		52.82 (± 5.81)

Supplementary Table II.3 - Analysis, by RNA-FISH and immunofluorescence, of the *FA-SAT* ncRNA volume/cell (µm3) in each cell cycle phase of HeLa cells. The RNA-FISH/IF images were captured through confocal microscopy and the volumes of the fluorescent signals (per cell) were measured, with specific software, in each phase of the cell cycle. A minimum of 10 cells was analyzed. Values are presented as mean ± SD.

		FA-SAT ncRNA volume/cell (µm3)
HeLa	G0 phase	6.17 (± 1.62)
	G1 / S transition	18.27 (± 3.10)
	S phase	12.09 (± 3.63)
	G2 phase	8.39 (± 3.02)
	Mitosis	4.20 (± 1.98)

CHAPTER III

FA-SAT ncRNA/PKM2 disruption: can P53 status dictate different outcomes?

Research article:

Lucas D, Ferreira D, Adega F, Chaves R. *FA-SAT* ncRNA/PKM2 regulates the cell-cycle progression in human cancer cells: is the P53 status a determining factor? *In preparation*.

III.1. Abstract

Satellite DNA sequences and their transcripts have seen their study largely boosted in the last years. *FA-SAT* was originally described as the major satellite DNA of the domestic cat genome, but now we know that it is highly conserved and transcribed in several phylogenetically distant species, including humans. In cat and human cells, it was stated that *FA-SAT* ncRNA can interact with the PKM2 (Pyruvate Kinase Muscle Isozyme) moonlighting protein, to form a ribonucleoprotein complex and to play nuclear functions in regulating the switch between cell proliferation and apoptosis. In this work, we aimed to explore how the *FA-SAT* ncRNA/PKM2 interaction influences the cell-cycle progression and apoptosis and we tried to unveil the P53 involvement in determining these outcomes. Using P53 wild-type and null cell lines, we carried out *FA-SAT* knockdown experiments and we analyzed the resulting cellular and molecular phenotypes. Then, it was observed that the *FA-SAT* ncRNA depletion leads to inhibition of cell proliferation (with *MYC* downregulation in both cell lines) and to apoptosis (with P53 accumulation in P53 wild-type cells). Moreover, when *FA-SAT* was silenced, it was detected a decrease in the *PKM2* RNA and nuclear protein levels in both cell lines. Thus, the data obtained attested to the existence of an association/interaction between *FA-SAT* ncRNA and the PKM2 protein, in these human cell lines, and showed the role of this ribonucleoprotein complex in defining the cell fate, regulating the mitogenic and apoptotic pathways and their key players. Finally, we suggest that these processes can occur independently of the P53 status, once similar phenotypes were observed, both in the P53 wild-type and null cells, when the *FA-SAT* ncRNA/PKM2 disruption was induced.

III.2. Introduction

Satellite DNAs (satDNAs) are a class of highly repetitive tandem sequences that are transcribed into non-coding RNAs (ncRNAs) and whose role in cell function and disease (e.g. cancer) has become increasingly accepted in the last years (reviewed in Ferreira et al. 2015; Louzada et al. 2020).

FA-SAT was primarily described, by Fanning (1987), as the major satDNA of the domestic cat *(Felis catus*). Nowadays we know it is preserved, with high similarity, in the genomes of several phylogenetically distant species including humans and, in the same species, it is also transcribed, yielding *FA-SAT* ncRNA (Chaves et al. 2017).

Ferreira and collaborators stated that this ncRNA can interact with the PKM2 (Pyruvate Kinase Muscle Isozyme) protein, forming a ribonucleoprotein complex with it, at the nucleus of cat and human proliferative cells. The *FA-SAT* knockdown, in these cells, resulted in a reduction of the *PKM2* RNA and protein levels (mainly in the nuclear PKM2) and, in turn, the *PKM2* silencing caused the decrease of the *FA-SAT* ncRNA levels. In both cases, the disruption of this complex led to the swift from cell proliferation to apoptosis. Moreover, the ectopic expression of this satDNA led to the PKM2 nuclear accumulation. Thus, it was suggested that the *FA-SAT* transcripts should be involved in the PKM2 recruitment to the nucleus, allowing it to perform its function(s) there (Ferreira et al. 2019).

In fact, PKM2 is considered a moonlighting protein once it acts as pyruvate kinase in the cytoplasm (Staal and Rijksen 1991) and as protein kinase when translocated to the nucleus (Harris et al. 2012; Iqbal et al. 2014). In this last cell organelle, it can interact with several targets, regulating different cellular and molecular processes such as gene expression (David et al. 2010; Yang et al. 2012), cell proliferation (Zhang et al. 2013; Wang et al. 2015), apoptosis (Xia et al. 2016), among others (Tamada et al. 2012; Dong et al. 2016).

Finally, it should be noted that the cellular phenotypes described in cat and human cells upon the *FA-SAT* ncRNA/PKM2 depletion, i.e., inhibition of cell proliferation and apoptosis, were accompanied by *MYC* downregulation and P53 accumulation. These are considered important key players of the mitogenic and apoptotic pathways, interacting with several targets, regulating them and defining the cell-cycle progression (reviewed in Bretones et al. 2015; Chen 2016). Moreover, in cancer cells, they are frequently altered contributing to tumorigenesis and cancer evolution (reviewed in Dang 2013; Sabapathy and Lane 2018). In fact, P53 is a key player of some of the main cell-cycle pathways/checkpoints (reviewed in Chen 2016; Sabapathy and Lane 2018) and its malfunction is implicated in different cancer hallmarks

(Hanahan and Weinberg 2000; Hanahan and Weinberg 2011), particularly the evasions to growth suppressors and to apoptosis.

For all these reasons, with this work, we intended to verify if the P53 status (presence or absence of this protein in the cells) can influence the *FA-SAT* silencing phenotypes, especially those related to the mitogenic and apoptotic pathways. For that, the *FA-SAT* knockdown was induced in P53 wild-type and null cells, and the resulting cellular and molecular (*MYC* RNA and P53 protein levels) outcomes were assessed. Also, the *FA-SAT* ncRNA/PKM2 interaction and its importance to cell function and disease, specifically in cell cycle (dys)regulation, were evaluated in the same cell lines. The data gathered here allowed us to better understand the mechanism(s) underlying the switch between cell proliferation and apoptosis of the *FA-SAT* ncRNA/PKM2 disruption.

III.3. Material and Methods

Cell culture and transfection

The A549 (P53 wild-type/WT, ATCC® CCL-185) cell line was grown in F12K medium, supplemented with 10% FBS (Fetal Bovine Serum), 1% of antibiotic mixture (50 µg/ml penicillin, 50 µg/ml streptomycin and 100 µg/ml neomycin) and 200 mM L-Glutamine. The H1299 (P53 null, ATCC® CRL-5803) cell line was grown in RPMI medium, supplemented with 15% FBS, 1% of antibiotic mixture (50 µg/ml penicillin, 50 µg/ml streptomycin and 100 µg/ml neomycin) and 200 mM L-Glutamine. For cell culture, routine procedures were applied and all the reagents are from Gibco (Thermo Fisher Scientific).

To perform the transfection assays, 300,000 cells/well were plated in a 6-well plate, with the specific medium without antibiotics and with 5% FBS. For each assay, three different mixtures were applied: 1) the mock control, containing only the transfection reagent, was used to exclude cytotoxicity; 2) the LNA negative control, containing 50 nM of an Antisense LNA™ GapmeR (Exiqon - Qiagen) with no phenotypic effect, was used to discard off-target effects; 3) the *FA-SAT* LNA, containing 50 nM of a customized Antisense LNA™ GapmeR (5′-FAM TGA TGC TGT CAG ACGT, Exiqon - Qiagen), was used to promote the *FA-SAT* knockdown (it hybridizes, by complementarity, with the *FA-SAT* RNA, promoting its degradation through RNase-H recruitment). The transfections were carried out using Lipofectamine® RNAiMAx Transfection Reagent (Invitrogen - Thermo Fisher Scientific) according to the manufacturer's instructions. After 4-6h, the medium was changed to the regular medium. The cells were

maintained in the incubator, at 37º C, 5% CO2 and 97% relative humidity, for 48h or 72h. For the *FA-SAT* depletion analysis, these cells were observed by microscopy 24, 48 and 72h after transfection and the respective images were captured. The success of the transfection experiments was proven by the observation of fluorescent signals in the cells, once the LNA molecules were labeled with the FAM fluorescent dye.

Similar experiments (with the same reagents, transfection mixtures and parameters) were carried out in cells grown on Superfrost Excell microscope slides (Thermo Scientific™), at a concentration of 100,000 cells/slide for immunofluorescence procedures.

RNA extraction

Total RNA was extracted from A549 and H1299 cells, 72h after the transfection using the mirVana Isolation Kit (Ambion - Thermo Fisher Scientific) and following the manufacturer's recommendations. Then, the RNA was purified using the TURBO DNA-free™ Kit (Ambion - Thermo Fisher Scientific) and it was quantified using the NanoDrop 1000 (Thermo Fisher Scientific).

Real-time RT-qPCR

The *FA-SAT*, *PKM2* and *MYC* expression was analyzed in the A549 and H1299 transfected cells, using the Verso 1-Step RT-qPCR kit, SYBR Green, ROX (Thermo Scientific) and following the manufacturer instructions. For that, the standard curve method (described in Chaves et al. (2017)) was applied (parameters presented in Supplementary Table III.1) and 100 ng of RNA were used in all the reactions

The *FA-SAT* RNA quantification was achieved using a pair of specific primers (Forward: AGCTAAGGCTCTCCCCTCATG; Reverse: TCAGCCTGCACCGCTTCT) and following the reaction: 50ºC (15 min); 95ºC (15 min); 40 cycles of 95ºC (15 sec), 59ºC (45 sec) and 72ºC (1 min). The remaining analyses were performed using a pair of specific primers for *PKM2* RNA (Forward: TAAGACCCTGTTCGGGTGAG; Reverse: GCTTCCAGTCCTGCAAACTC) and another one for *MYC* RNA (Forward: AGCAAACCTCCTCACAGCCC; Reverse: ACTGTCCAACTTGACCCTCT). For the quantification of the expression of both *PKM2* and *MYC* genes, the next reaction was followed: 50ºC (15 min); 95ºC (15 min); 40 cycles of 95ºC (15 sec) and 60ºC (1 min).

To evaluate the primers' specificity, a melt curve was obtained in the different reactions. Replicates and negative controls (without RNA) were used to increase the precision and

accuracy of the results. All the experiments were performed in a StepOne real-time PCR system (Applied Biosystems - Thermo Fisher Scientific) and the data were analyzed with the StepOne software (version 2.2.2, Applied Biosystems - Thermo Fisher Scientific).

MitoTracker

A MitoTracker® Red CMXRos (Invitrogen - Thermo Fisher Scientific) protocol was applied to A549 and H1299 transfected cells grown on microscope slides (Superfrost Excell, Thermo Scientific™), for mitochondria labeling and apoptosis detection. The cells were, firstly, incubated with 50 nM MitoTracker® probe solution in HBSS, for 15 min at 37ºC. Then, they were fixed with 2% (m/v) paraformaldehyde in PBS, for 20 min and mounted and counterstained with Vectashield mounting medium (VectorLaboratories) containing 4'-6-diamidino-2- phenylindole (DAPI).

Immunofluorescence

An immunofluorescence experiment was developed using A549 and H1299 cells (grown and transfected on Superfrost Excell microscope slides), in order to detect and (semi)quantify the PKM2 and Phospho-P53 (ser15) proteins. Initially, cell fixation was achieved using 2% (m/v) paraformaldehyde in PBS for 20 min, followed by their permeabilization with 4% (v/v) Triton X-100 in PBS for 15min. Blocking was performed with 10%FBS in PBST for 30 min. Primary antibody labeling was performed for 1h with: anti-phospho-P53 (ser15) monoclonal antibody C.381.0 (1:100, mouse, Invitrogen - Thermo Fisher Scientific MA5-15229); or anti-PKM2 polyclonal antibody (1:50, rabbit, Merck Millipore ABS245). After that, the detection with the secondary antibody was carried out for 1h: anti-mouse polyclonal antibody FITC conjugate (1:200, Zymed - Thermo Fisher Scientific 81-6511); or anti-rabbit polyclonal antibody FITC conjugate (1:200, Sigma-Aldrich - Merck Millipore MAP132F). Lastly, the cells were mounted and counterstained using Vectashield mounting medium (VectorLaboratories) with DAPI.

Microscopy and Image analysis

For analysis of the cellular phenotypes resulting from the cell transfection/*FA-SAT* knockdown, a Zeiss Axiovert 200 microscope and the P.A.L.M. software were used.

The MitroTracker and IF images were captured through confocal microscopy, using a Zeiss Axio Imager Z1 microscope/LSM 510 META and the respective LSM 510 software (version

4.0 SP2). A group of three lasers (argon 488nm, helium-neon 543nm and diode 405nm) was used to capture all the images and the microscope settings were maintained to normalize the results: argon laser set at 12.9% (pinhole of 96mm/1.02 airy units), helium-neon laser at 50.8% (pinhole of 102 mm/0.98 airy units) and diode laser at 9.9% (pinhole of 112mm); 63x objective; thickness of the Z sections of 1 µm; scan speed of 4.

The confocal microscopy images were then analyzed and treated with different software's: AutoQuant X3 (Media Cybernetics) for deconvolution; ImageJ 1.53c (National Institutes of Health) to merge the color channels and to generate a 2D projection of the different stacks of Z-axis, creating a single and representative TIFF image; Image Pro 10.0.5 (Media Cybernetics) to count and measure volumes of the fluorescent signals, through the creation of 3D Iso-Surfaces.

All the presented images were also optimized (at whole image) for color, brightness and contrast, using Adobe Photoshop CC 2019.

Statistics

All data were analyzed and the statistical significance was determined by two-tailed Student's t-test (to compare two independent samples) or one-way ANOVA test (to evaluate the association between more than two independent samples), as mean ± standard deviation (SD): non-significant or ns ($p > 0.05$); * ($p \leq 0.05$); ** ($p \leq 0.01$); *** ($p \leq 0.001$); **** ($p \leq 0.0001$).

III.4. Results

Earlier, Ferreira and colleagues had stated that this ncRNA can interact with the PKM2 protein to perform nuclear functions in cat and human (HeLa) cells, namely regulating the switch between cell proliferation and apoptosis (Ferreira et al. 2019). Thus, in this work, we performed *FA-SAT* silencing experiments and we investigated the resulting cellular and molecular outcomes, to understand the contribution of *FA-SAT* ncRNA/PKM2 to the cell cycle of the A549 (P53 WT) and H1299 (P53 null) human cancer cells. With this approach, we aimed to unveil the influence of the P53 status (presence or absence of this protein in the cells) on these phenotypes.

FA-SAT knockdown triggers the switch between cell proliferation and apoptosis, both in P53 WT and P53 null cells

Functional assays for the *FA-SAT* silencing were carried out in P53 WT and null cells with a customized antisense LNA™ GapmeR, as previously described by Ferreira et al. (2019). The success of the knockdown in these cells was achieved 72h after transfection (Figure III.1a, Supplementary Table III.2) with significant decreases of the *FA-SAT* expression (74% in A549 cells and 81% in H1299 cells). Other conditions were analyzed (48h), but the *FA-SAT* RNA levels did not show significant differences between *FA-SAT* LNA and control samples (Supplementary Figure III.1, Supplementary Table III.2).

The cellular phenotypes resulting from the *FA-SAT* ncRNA depletion were investigated through microscopic observation 24h, 48h and 72h after the transfection with the different mixtures. In Figure III.1b, it was possible to verify that in the control samples (mock and CTR neg) there was an increase in the number of cells over the 72h, demonstrating a normal cell proliferation of the A549 and H1299 cells. In contrast, in the cells transfected with the *FA-SAT* LNA, it was observed an increase in cell death, also in the two cell lines analyzed. Furthermore, the cells successfully transfected (shown with green fluorescence in Figure III.1b) are the ones that died. Then, it was established that the *FA-SAT* knockdown results in inhibition of cell proliferation and promotion of cell death, both in P53 WT and null cells.

In order to understand if the cell death observed after *FA-SAT* depletion is apoptosis, it was performed the mitochondria staining with MitoTracker assay in both A549 and H1299 cells, 72h after their transfection. In Figure III.1c, it can be observed a decrease in the signal of MitoTracker fluorophore in the *FA-SAT* silenced cells, in both cell lines, indicating a lower mitochondrial membrane potential in these cells, when compared with the mock and negative controls. These differences can be explained by the occurrence of changes in the mitochondrial permeability, which are necessary for cytochrome c release and activation of the intrinsic apoptotic pathway (Poot et al. 1997; Green and Reed 1998; Pendergrass et al. 2004), in the cells where *FA-SAT* was silenced. These results indicate that the cell death observed in the *FA-SAT* ncRNA depleted cells is apoptosis in both cell lines.

It is also noteworthy that, during the 72h experiment, no differences were found between the mock and CTR neg samples, so, in the next results, only the mock data will be shown (and considered as control) in order to simplify the analysis.

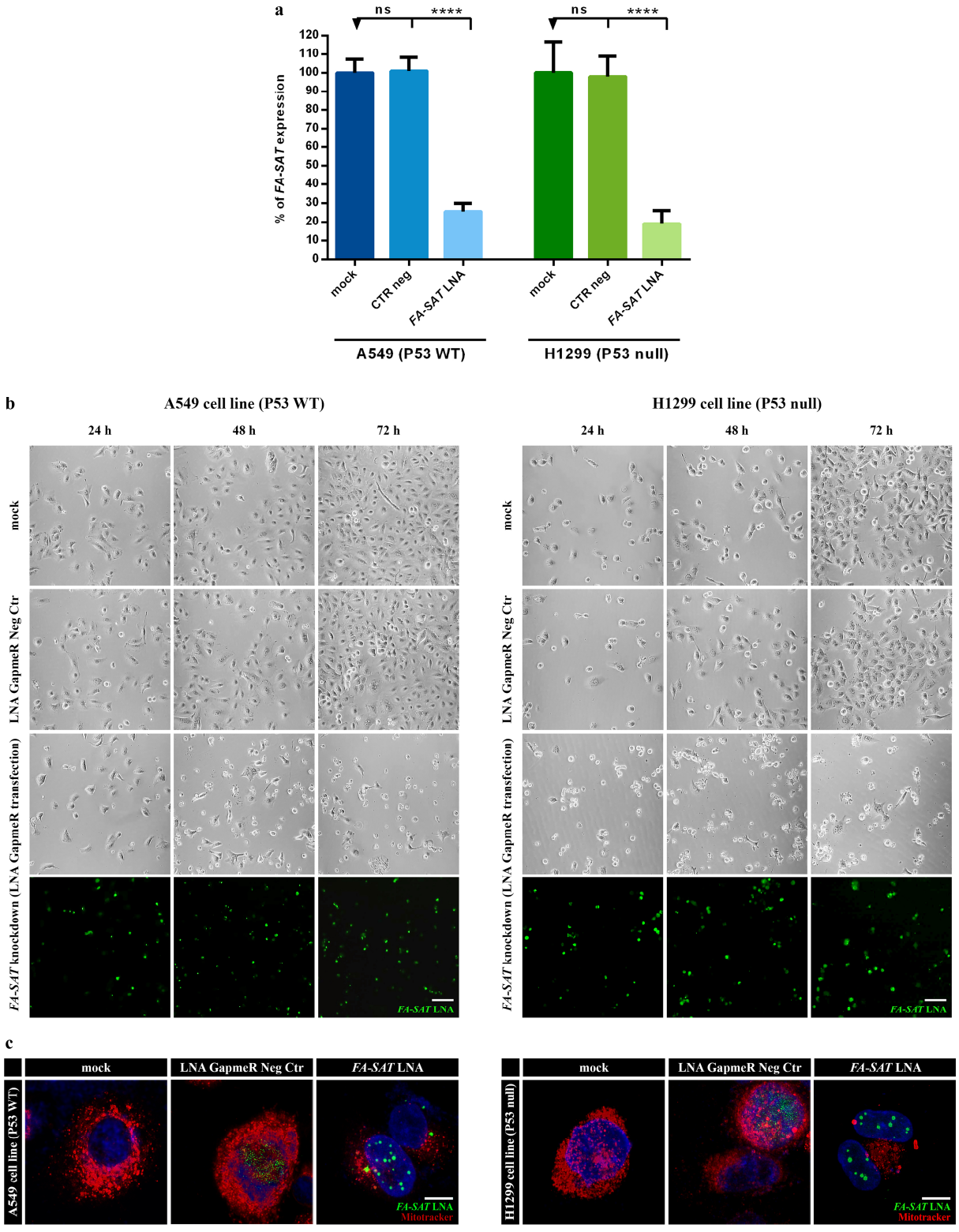


Figure III.1 - *FA-SAT* knockdown leads to inhibition of cell proliferation and apoptosis both in the A549 (P53 WT) and H1299 (P53 null) cells. (a) Analysis, by RT-qPCR, of the *FA-SAT* expression (%) in the A549 and H1299 transfected cells. Mock and CTR neg mixtures were used as a reference. The statistical significance was determined by one-way ANOVA. Values are presented as mean ± SD: non-significant or ns ($p > 0.05$); **** ($p \leq 0.0001$). **(b)** Cell imaging of the A549 and H1299 cells, 24, 48 and 72 h after the transfection. The LNA GapmeR was FAM labeled (green). The scale bar represents 100 µm. **(c)** Mitochondrial staining (red, MitoTracker fluorophore) and apoptosis detection in the A549 and H1299 cells, 72h after the transfection with the mock, CTR neg and *FA-SAT* LNA (green) mixtures. Scale bar represents 10 µm. The DNA was stained with DAPI (blue).

Finally, since similar cellular phenotypes were observed both in the P53 WT (A549) and the P53 null (H1299) cells, we suggested that these processes might occur independently of the P53 status. In order to confirm this hypothesis and to access the molecular outcomes resulting from *FA-SAT* knockdown, specifically those related to the proliferative and apoptotic pathways, we examined then the expression levels of *MYC* and *P53*.

FA-SAT knockdown interferes with the mitogenic and apoptotic pathways interrelating with their key players: the P53 absence does not compromise the resulting phenotypes

MYC and *P53* are considered major key players of the mitogenic and apoptotic pathways, respectively, controlling some of their subjacent mechanisms and, in the last instance, defining the cell fate (reviewed in Bretones et al. 2015; Chen 2016). In this part of the work, we evaluated if their expression levels could be affected by the *FA-SAT* knockdown and how could this be related to the cellular phenotypes described above.

The *MYC* RNA was quantified, by RT-qPCR, in P53 WT and null cells in which *FA-SAT* silencing was induced (Figure III.2 and Supplementary Table III.3). Comparing the mock and *FA-SAT* LNA samples of each cell line, we realized that, in the last ones, there was a significant reduction of the *MYC* RNA levels of approximately 74% in A549 cells and 64% in H1299 cells. Thereby, we defined that the *FA-SAT* knockdown results in *MYC* downregulation, in both cell lines analyzed, agreeing with the cellular phenotype (inhibition of cell proliferation).

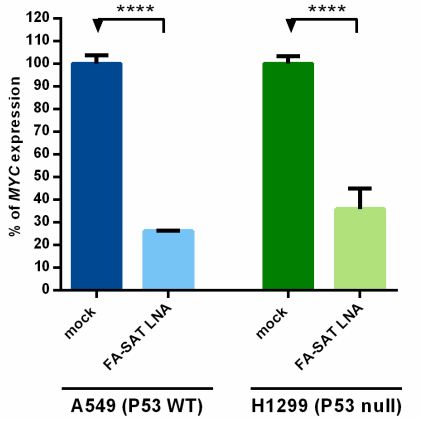


Figure III.2 - _FA-SAT_ silencing results in a decrease of the _MYC_ expression, both in the P53 WT and P53 null cell lines. Analysis, by RT-qPCR, of *MYC* RNA levels (%) in the A549 and H1299 cells, 72h after their transfection with mock and *FA-SAT* LNA. Mock mixtures were used as a reference. The statistical significance was determined by two-tailed Student's t-test. Values are presented as mean ± SD: **** ($p \leq 0.0001$).

The assessment of the P53 levels in the P53 WT and null transfected cells enabled us to understand the importance of this protein in determining the observed cell-cycle arrest and apoptosis phenotypes. More precisely, we quantified the amount of phospho-P53 (ser 15), once

the phosphorylation at serine 15 is fundamental for P53 activation and function in cell-cycle arrest and/or apoptosis (Shieh et al. 1997; Loughery et al. 2014; Yang et al. 2019). Thus, the observation of microscopy images (Figure III.3a) and the respective quantification of the IF signals (Figure III.3b and Supplementary Table III.4) revealed that the *FA-SAT* silencing results in the phospho-P53 (ser 15) accumulation in the A549 cells, suggesting the involvement of this protein in defining the mentioned cellular phenotypes. However, a similar analysis confirmed the absence of the P53 protein in the H1299 cells (Figure III.3 and Supplementary Table III.4) and the same cellular phenotypes had been reported for them.

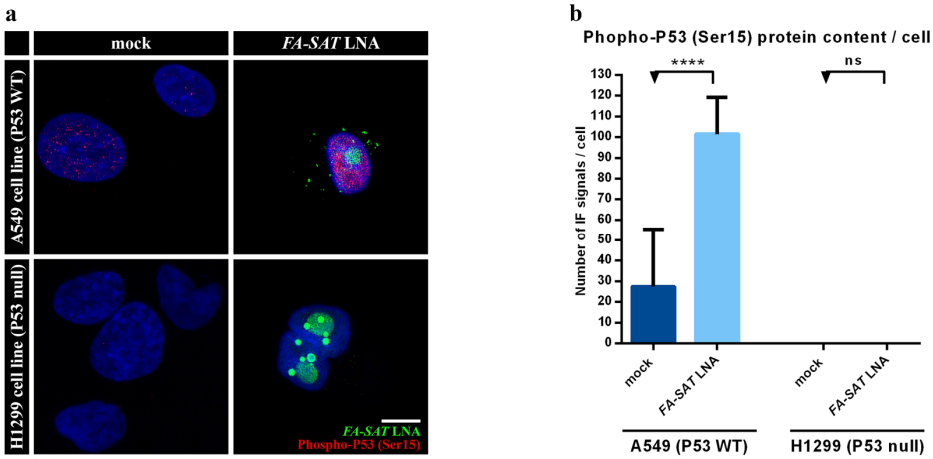


Figure III.3 - Phospho-P53 (Ser15) accumulates in A549 cells as a consequence of the *FA-SAT* knockdown and it is absent in H1299 cells. (a) IF detection of the Phospho-P53 (Ser15) protein (red) in the A549 and H1299 cells, 72h after their transfection with mock and *FA-SAT* LNA (green). The DNA was stained with DAPI (blue). Scale bar represents 10 µm. **(b)** Quantification of the Phospho-P53 (Ser15) content/cell (number of IF signals) in the A549 and H1299 transfected cells. A minimum of 10 cells was analyzed. Mock mixtures were used as reference. The statistical significance was determined by two-tailed Student's t-test. Values are presented as mean ± SD: non-significant or ns ($p > 0.05$); **** ($p \leq 0.0001$).

PKM2 expression is positively correlated with the *FA-SAT* ncRNA levels: their interaction should be crucial for determining the cell fate in human cancer cells

PKM2 is a moonlighting protein (Iqbal et al. 2014) that plays cellular functions in the regulation of processes such as gene expression, cell proliferation and apoptosis, among others (Tamada et al. 2012; Dong et al. 2016). Since Ferreira et al. (2019) had reported that *FA-SAT* ncRNA can interact with this protein in cat and human HeLa cells, we tried to understand if, in the A549 and H1299 human cell lines, this association is also verified and how could it be linked with the cellular and molecular phenotypes described up to this point.

So, the *PKM2* expression was examined through cell imaging technologies and molecular analysis in *FA-SAT* depleted cells from both cell lines. In the *FA-SAT* LNA samples, a reduction

in the PKM2 protein was detected, specifically at the nucleus (Figure III.4a), from approximately 54 to 14 signals/nucleus in the A549 cells and from 79 to 23 signals/nucleus in the H1299 cells (Figure III.4b and Supplementary Table III.5). In the same conditions, the *PKM2* RNA amount, analyzed by RT-qPCR, showed to be decreased: 58% in the P53 WT cell line and 94% in P53 null cells (Figure III.4c and Supplementary Table III.6).

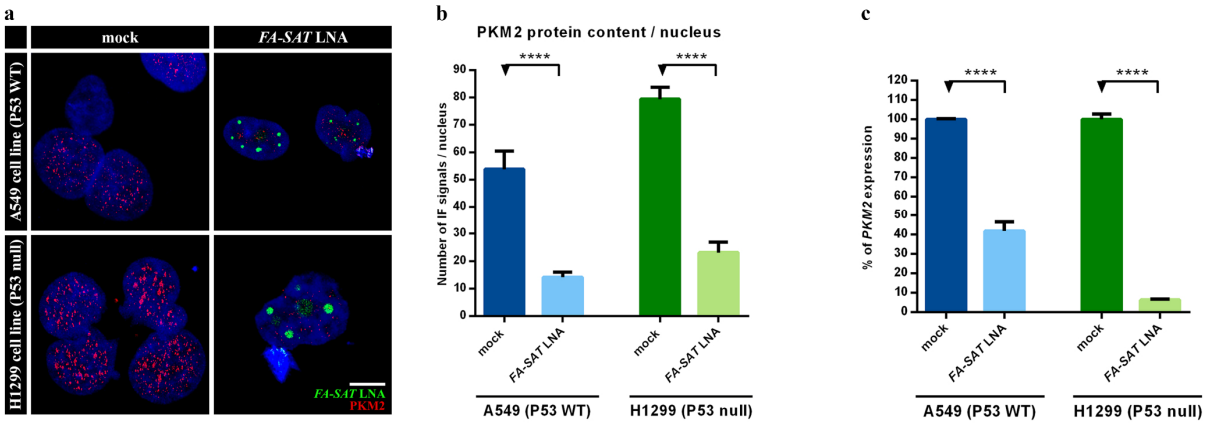


Figure III.4 - *FA-SAT* depletion induces *PKM2* downregulation in A549 and H1299 cells. (a) IF detection of the PKM2 protein (red) in A549 and H1299 cells transfected with mock and *FA-SAT* LNA (green). The DNA was stained with DAPI (blue). Scale bar represents 10 µm. **(b)** Quantification of the PKM2 content/nucleus (number of IF signals) in the A549 and H1299 transfected cells. A minimum of 10 cells was analyzed. **(c)** Quantification, by RT-qPCR, of the *PKM2* RNA (%) in A549 and H1299 transfected cells. Mock mixtures were used as reference. The statistical significance was determined by two-tailed Student's t-test. Values are presented as mean ± SD: **** ($p \leq 0.0001$).

These data demonstrated the existence of an association/interaction between *FA-SAT* ncRNA and the PKM2 protein in the nucleus of the A549 and H1299 cells, being this complex probably involved in the regulation of cell proliferation and apoptosis mechanisms previously presented.

III.5. Discussion

In recent years, it was discovered that *FA-SAT* is highly conserved and transcribed in human species, yielding *FA-SAT* ncRNA (Chaves et al. 2017). This ncRNA plays nuclear functions in regulating the cell-cycle progression and its depletion results in apoptosis (with P53 accumulation), both in cat and human cells (Ferreira et al. 2019). Thus, we performed a series of functional assays inducing the *FA-SAT* knockdown in P53 WT and null cell lines, to ascertain if the P53 status can influence the *FA-SAT* silencing outcomes (either in terms of the

FA-SAT ncRNA/PKM2 interaction or regarding its ability to cross-talk between the mitogenic and apoptotic pathways).

Primarily, we observed that the depletion of this ncRNA resulted in the impairment of cell-cycle progression (the number of cells did not increase over time as it happens in the control samples) and in a reduction of the *MYC* RNA levels, both in A549 and H1299 cell lines. In other works, the transcriptional activity of some satDNAs, such as the human satellite II, had been linked to cell proliferation and its knockdown was pointed as a way to suppress the abnormal cell division process of cancer cells (Bersani et al. 2015; Nogalski and Shenk 2020). Also, similar results were previously reported by Ferreira et al. (2019) in *FA-SAT* depleted cat and other human cells. Besides, considering the *MYC* crucial role in controlling the cell-cycle progression through several pathways (reviewed in Dang 2013; Bretones et al. 2015), the results obtained in the molecular analysis of *MYC* expression were in concordance with the cellular phenotypes observed: by unknown mechanisms, the decrease in *FA-SAT* ncRNA levels should lead to *MYC* downregulation and, consequently, to the inhibition of cell proliferation.

Upon *FA-SAT* silencing, it was also observed an increase in cell death, specifically apoptosis, both in P53 WT and null cells. Several authors have reported cell-cycle arrest/apoptosis phenotypes associated with the depletion of some satellite ncRNAs, for example the human alpha satellite (McNulty et al. 2017) and satellite III (Goenka et al. 2016) transcripts. Also, as previously referred, the phenotype observed in *FA-SAT* silenced cat and human cells was apoptosis with an accumulation of P53 (Ferreira et al. 2019). Since P53 is extensively involved in some of the main cell-cycle pathways/checkpoints (reviewed in Chen 2016; Sabapathy and Lane 2018) and its phosphorylation at serine 15 is fundamental for its activation and function in cell-cycle arrest and/or apoptosis (Shieh et al. 1997; Loughery et al. 2014; Yang et al. 2019), we decided to assess the phospho-P53 (ser 15) protein levels, to reveal the putative associations between the *FA-SAT* silencing and the P53 status. Then, in A549 *FA-SAT* depleted cells, in which the apoptosis phenotype was observed, it was detected a significant accumulation of phospho-P53 (ser 15), suggesting its involvement in mediating/triggering this outcome. Nevertheless, the phospho-P53 (ser 15) absence was confirmed in the H1299 cells when *FA-SAT* was silenced, but the activation of apoptotic pathway(s) had also been noticed in them. With this information, we stated that this phenotype is P53-independent in the H1299 cells and should exist in them an alternative cell pathway(s) that may lead to the same outcome. Previously, Chang and colleagues had reported that the chemotherapeutic agent gemcitabine induces P53-independent apoptosis in H1299 cells through ERK activation and Akt

inactivation, promoting the downregulation of the Bcl-2 and Bcl-X_L anti-apoptotic proteins (Chang et al. 2004). In another study, it was discovered that the phytochemical garcinol can trigger the p38-MAPK signaling inactivation and, consequently, the $p21^{Waf1/Cip1}$ upregulation (CDK inhibitor), stimulating the G1 cell-cycle arrest in the same cell line (Yu et al. 2014). In our work, the *FA-SAT* knockdown showed to be certainly associated with the cell-cycle arrest/apoptosis of the P53 WT and null cells, however, the process by which this is determined needs to be further investigated.

The analysis of the PKM2 levels in the A549 and H1299 transfected cells let us establish a correspondence between the *FA-SAT* knockdown and a reduction in the amount of this protein (also its RNA), specifically at the nucleus. Thus, we attested the existence of a nuclear association between *FA-SAT* ncRNA and this kinase in these human cell lines. Furthermore, considering the functions described for PKM2 in cell-cycle regulation (reviewed in Tamada et al. 2012), this ribonucleoprotein interaction should have had a fundamental role in determining the cellular and molecular phenotypes described above. In fact, the PKM2 knockdown has already been linked to inhibition of cell proliferation (Ao et al. 2017) and reduced expression of pro-mitogenic genes, including *MYC* and *Cyclin D1* (Lü et al. 2013; Yang et al. 2016). Its silencing was also associated with cell-cycle arrest/apoptosis induction (Goldberg and Sharp 2012; Ao et al. 2017), increased activity of arrest agents, e.g., p21 (Ao et al. 2017), downregulation of anti-apoptotic proteins, such as Bcl-2 (Chu et al. 2015; Yang et al. 2016) and Bcl-X_L (Kwon et al. 2012), and upregulation of proteins with pro-apoptotic activity: P53 (Ao et al. 2017), BAD (Miao et al. 2016), BAX (Yang et al. 2016) and BIM (Hu et al. 2015).

In short, with the results here obtained, we could conclude that in the A549 and H1299 human cells, not only *FA-SAT* ncRNA interacts with PKM2 protein, but they should be responsible for, together, regulating the shift from cell proliferation to apoptosis, interrelating with the key players of these cell pathways. All these findings corroborate the information reported by Ferreira et al. (2019) for cat and human cells, indicating the existence of conserved functions for these transcripts between phylogenetically distant species (cat and human) and between different cells/tissues of the same species (HeLa - cervix; A549 and H1299 - lung).

Finally, and answering the central question of this study, we hypothesize that the *FA-SAT* knockdown phenotypes are independent of the P53 status, that is, they can involve P53 mediation or occur through alternative pathways. Hereafter, this premise should be further explored to unveil the mechanism(s) by which this complex can regulate the cell-cycle progression, in a P53-dependent or -independent way.

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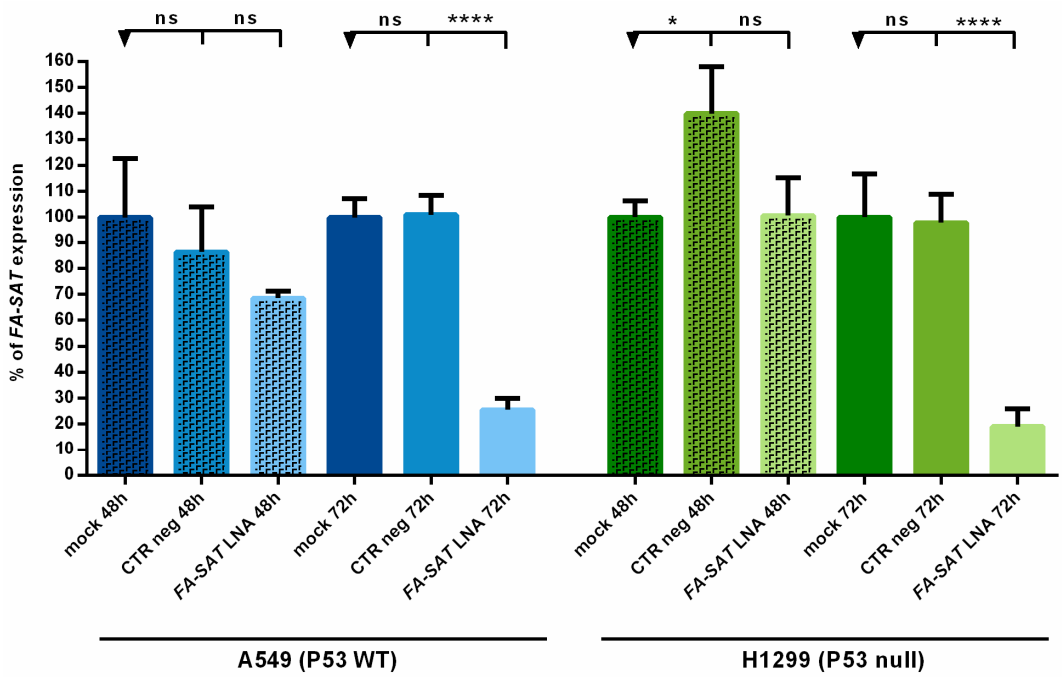
Supplementary information

Supplementary figures



Supplementary Figure III.1 - Comparative analysis, by RT-qPCR, of the *FA-SAT* expression (%) in the A549 and H1299 transfected cells (mock, CTR neg and *FA-SAT* LNA), to infer the efficiency of transfection at 48h and 72h. Mock mixtures were used as reference. The statistical significance was determined by one-way ANOVA. Values are presented as mean ± SD: non-significant or ns ($p > 0.05$); * ($p \leq 0.05$); **** ($p \leq 0.0001$).

Supplementary tables

Supplementary Table III.1 - Standard curve parameters

	R^2	Efficiency (%)
FA-SAT RNA	0.99	98.23
PKM2 RNA	0.99	110.00
MYC RNA	0.99	97.39

Supplementary Table III.2 - Analysis, by RT-qPCR, of the *FA-SAT* expression (%) in the A549 and H1299 cells, 48h and 72h after their transfection with mock, CTR neg and *FA-SAT* LNA mixtures. Mock mixtures were used as reference. Values are presented as mean ± SD.

			FA-SAT expression (%)
A549 (P53 WT)	48h	mock	100.00 (±22.74)
		CTR neg	86.28 (± 17.66)
		FA-SAT LNA	68.47 (± 2.72)
	72h	mock	100.00 (± 7.26)
		CTR neg	101.00 (± 7.41)
		FA-SAT LNA	25.59 (± 4.26)
H1299 (P53 null)	48h	mock	100.00 (± 6.33)
		CTR neg	139.90 (± 18.08)
		FA-SAT LNA	100.70 (± 14.47)
	72h	mock	100.00 (± 16.75)
		CTR neg	97.96 (± 10.98)
		FA-SAT LNA	19.08 (± 6.92)

Supplementary Table III.3 - Analysis, by RT-qPCR, of the *MYC* expression (%) in the A549 and H1299 cells, 72h after their transfection with mock and *FA-SAT* LNA mixtures. Mock mixtures were used as reference. Values are presented as mean ± SD.

			MYC expression (%)
A549 (P53 WT)	72h	mock	100.00 (± 3.68)
		FA-SAT LNA	26.16 (± 0.05)
H1299 (P53 null)	72h	mock	100.00 (±3.28)
		FA-SAT LNA	35.83 (± 8.98)

Supplementary Table III.4 - Relative quantification of the Phospho-P53 (Ser15) protein signals/cell in the A549 and H1299 transfected cells (mock and *FA-SAT* LNA). A minimum of 10 cells was analyzed. Mock mixtures were used as reference. Values are presented as mean ± SD.

			Phospho-P53 Ser 15 signals/cell
A549 (P53 WT)	72h	mock	27.40 (± 27.96)
		FA-SAT LNA	101.40 (± 17.79)
H1299 (P53 null)	72h	mock	0.00 (±0.00)
		FA-SAT LNA	0.00 (± 0.00)

Supplementary Table III.5 - Relative quantification of the PKM2 protein signals/cell in the A549 and H1299 transfected cells (mock and *FA-SAT* LNA). A minimum of 10 cells was analyzed. Mock mixtures were used as reference. Values are presented as mean ± SD.

			PKM2 signals/cell
A549 (P53 WT)	72h	mock	53.80 (± 6.60)
		FA-SAT LNA	14.10 (± 1.85)
H1299 (P53 null)	72h	mock	79.40 (± 4.40)
		FA-SAT LNA	23.10 (± 3.73)

Supplementary Table III.6 - Analysis, by RT-qPCR, of the *PKM2* expression (%) in the A549 and H1299 cells, 72h after their transfection with mock and *FA-SAT* LNA mixtures. Mock mixtures were used as reference. Values are presented as mean ± SD.

			PKM2 expression (%)
A549 (P53 WT)	72h	mock	100.00 (± 0.26)
		FA-SAT LNA	41.78 (± 4.73)
H1299 (P53 null)	72h	mock	100.00 (±2.64)
		FA-SAT LNA	6.24 (± 0.40)

CHAPTER IV

General discussion and concluding remarks

Over the last few years, satDNAs have gained some prominence and interest from the scientific community, as they have been associated with some key roles in the regulation of cell function and disease. This type of sequences, generally, are not well conserved between distant taxa, however, under certain conditions, they can remain "frozen" in the genomes along the evolutionary process, reflecting the conservation of fundamental functions (Ugarkovic 2005; Biscotti et al. 2015; Chaves et al. 2017; Ferreira 2018). *FA-SAT*, the major satDNA of the domestic cat genome, is an example of it. It was firstly discovered and studied in this species, due to the central role it plays in its genome (Fanning 1987), but currently, it is known that *FA-SAT* is highly conserved and transcribed (into ncRNA) in several Bilateria species, including humans (Chaves et al. 2017). Ferreira and colleagues carried out several studies to describe the *FA-SAT* transcriptional profile and to find its function(s), having stated: 1) its nuclear location in all the cat cells; 2) its cell-cycle dependent expression in cat non-tumor cells and the dysregulation of it in the tumor ones; 3) the *FA-SAT* ncRNA/PKM2 interaction, capable of regulating the swift between cell proliferation and apoptosis in cat and human cells (Ferreira 2018; Ferreira et al. 2019a).

In this sense, with this work, we performed the cellular characterization of *FA-SAT* ncRNA in distinct human cancer cells (Chapter II), for the first time. Our findings, namely the preservation of the ncRNA nuclear location in all the cell lines analyzed (HeLa, A549 and H1299) and its distribution throughout the cell cycle of HeLa cells, were essential to define its cellular profile and to establish the link with the data previously gathered from the cat species. This information was also used to proceed to the functional study of this ncRNA in humans, in Chapter III. The *FA-SAT* ncRNA/PKM2 association and the inherent regulation of the mitogenic and apoptotic pathways were scrutinized and compared with the previous results from cat. Furthermore, considering the P53 accumulation was earlier associated with the apoptosis phenotype triggered by the *FA-SAT* ncRNA/PKM2 disruption (Ferreira et al. 2019a), we investigated its implication in this outcome, using the A549 (P53 WT) and H1299 (P53 null) cells.

This work represents an advance in knowledge, so far as it contributes to understanding how the differential expression of this ncRNA (and its interaction with some targets) can modulate the cell-cycle progression and the cell fate in humans. The way in which it offers an upgrade for the state of the art related to *FA-SAT* (ncRNA) is schematized in Figure IV.1.

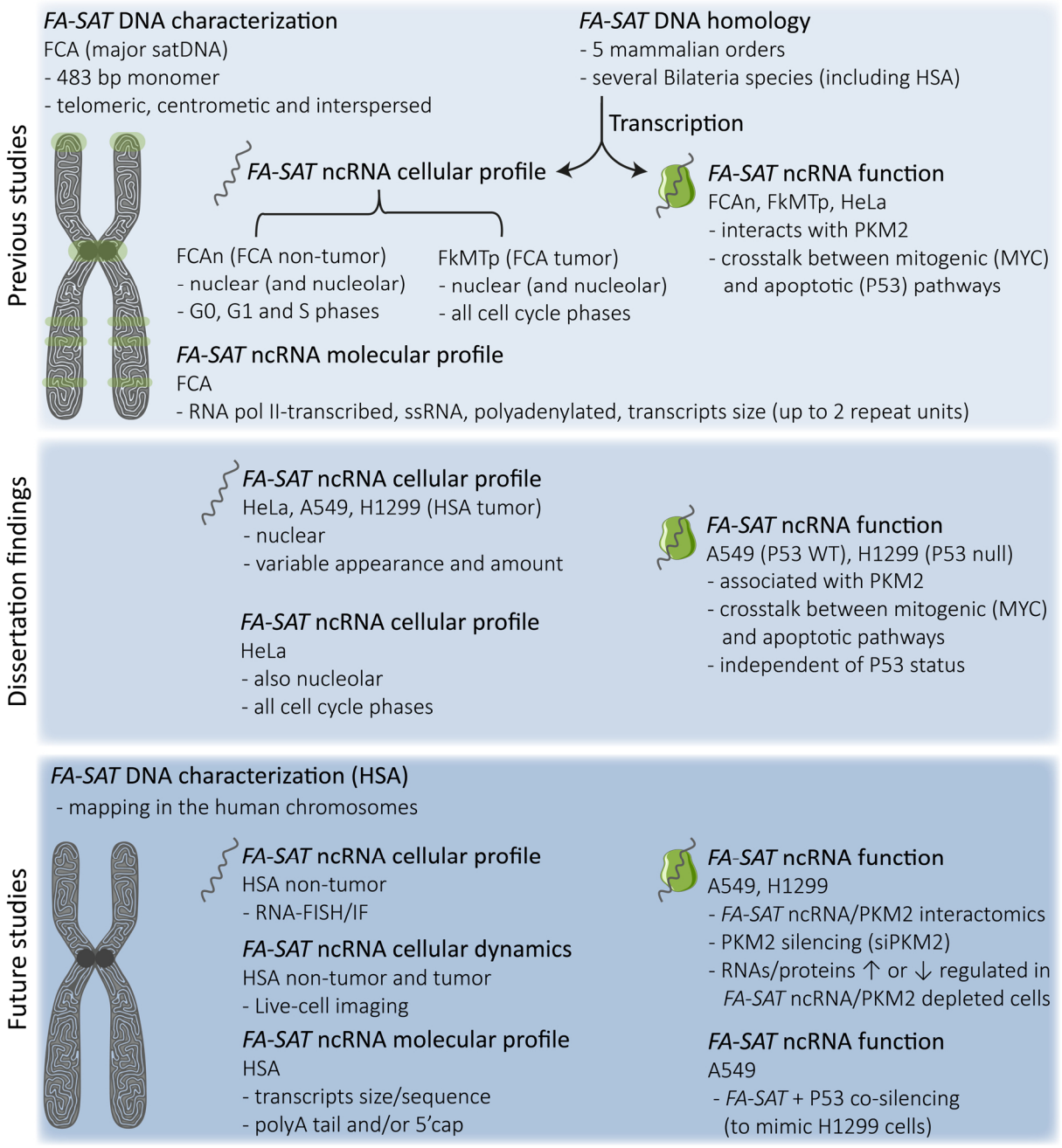


Figure IV.1 - Current state of the art related to *FA-SAT* (ncRNA) in the different species and research lines to be followed in the future. This integrative summary is divided into three interrelated sections: previous studies on the *FA-SAT* DNA characterization/conservation, description of its ncRNA profile and functional analysis of it in the cat (FCA) and human (HSA) species; data acquired throughout this work, concerning the ncRNA profile and functionality in human cells; some of the further steps that should be taken to complement the knowledge obtained so far in human species.

IV.1. The amount and appearance of *FA-SAT* ncRNA vary across human cell lines, but its location in the cell is preserved

In Chapter II, the *FA-SAT* ncRNA profile was described, for the first time, in different human cancer cells (HeLa, A549 and H1299), comparing its amount, organization and location in them. Regarding the amount of transcripts, significant differences were found across cell lines, both through molecular and single-cell analysis methods: H1299 cell line showed to be the one with higher levels of *FA-SAT* transcripts and the HeLa cells presented the lowest levels. Concerning the ncRNA organization in the cells, distinct possible arrangements were also observed, predominating the clustered fashion in HeLa and A549 nuclei and the scattered in the H1299 nuclei. These variations of the *FA-SAT* ncRNA behavior had already been reported for cat cells (Ferreira 2018; Ferreira et al. 2019a; Ferreira et al. 2019b) and some authors had stated that the satncRNAs expression can be modified depending on extrinsic and/or intrinsic factors, such as the cell type/line (Bury et al. 2020), the exposure to environmental stress conditions (Jolly et al. 2003) and in cancer (Ting et al. 2011).

Despite these fluctuations, one of the features remained unchanged among the different types of cells, i.e., the nuclear location of the transcripts in all the cell lines under study, pointing to the conservation of the nuclear function of these transcripts. Yet, an additional analysis involving HeLa cells revealed the presence of this ncRNA in a specific nuclear compartment, the nucleolus (and its periphery). This spatial distribution was previously reported for *FA-SAT* ncRNA, in cat cells (Ferreira et al. 2019a), and for other satncRNAs (Wong et al. 2007; Vautrot et al. 2015; Bury et al. 2020), as an indicator of the regulatory functions they play in this/these cellular compartment(s).

Moreover, the similarities found in the *FA-SAT* ncRNA profile between cat and human cells, highlight the preservation of its transcriptional features in these phylogenetically distant species.

IV.2. *FA-SAT* ncRNA distribution in human cells anticipates its nuclear functions in some phases of the cell cycle

Still in Chapter II, the *FA-SAT* expression was studied along the cell division process of the HeLa cell line, through the analysis of RNA-FISH/IF microscopy images and measure of the volume of transcripts per cell, in each phase of the cell cycle. Primarily, we noticed the

presence of this ncRNA in all the cell cycle phases of the HeLa cells, similarly to what had been reported for cat tumors (suggesting that the expression of this satDNA should be dysregulated in these human cancer cells as it happens in the cat ones), but contrary to that observed in cat non-tumor cells (Ferreira 2018; Ferreira et al. 2019a). Some of the hypotheses that may explain the presence of the *FA-SAT* transcripts across all the cell cycle of the HeLa cells are: 1) the maintenance of this ncRNA may be necessary to perform additional functions in these cancer cells; 2) the *FA-SAT* ncRNA turnover mechanisms may have been compromised in these cells, resulting in its accumulation; 3) the RNA-FISH methodology used in this work (with signal amplification system) allowed, with better resolution, to detect transcripts even when their levels were lower.

Notwithstanding, the quantification of the *FA-SAT* ncRNA volume/cell enabled us to detect variations in its amount over the cell cycle, with some accumulation in the late G1 and G1/S transition (highlighting the idea that its transcription may be, in fact, cell-cycle dependent). This type of temporal distribution of the transcripts was also reported for *FA-SAT*, in cat cells (Ferreira 2018; Ferreira et al. 2019a), and for other satDNAs, by Lu and Gilbert (2007) and Bury et al. (2020).

Since there is a consistent nuclear location of *FA-SAT* ncRNA in all the cell lines analyzed and it seems to exist an enrichment of these transcripts mainly in the G1 and S phases (in both cat and human species), it can be suggested that there are conserved functions played by this ncRNA at this cell compartment and in these phases of the cell cycle.

IV.3. *FA-SAT* ncRNA/PKM2 regulates the cell cycle, cross-talking between the mitogenic and apoptotic pathways, in human cells

After characterizing the *FA-SAT* ncRNA profile in different human cell lines, including the A549 and the H1299 (Chapter II), we proceeded to its functional study in these cells, in Chapter III. Thus, we induced the *FA-SAT* knockdown (through the transfection with a customized Antisense LNA™ GapmeR) and examined its cellular and molecular outcomes, either in terms of the *FA-SAT* ncRNA/PKM2 interaction or its impact in cell proliferation and apoptosis. All this analysis was performed in both P53 WT (A549) and null (H1299) cells, to compare the resulting phenotypes in each cell line and infer about the interference of the P53 status in them (the conclusions of it will be further discussed in the Subchapter IV.4).

The evaluation of the PKM2 amount in the *FA-SAT* silenced cells enabled us to establish an association between the knockdown of this satDNA and a decrease in the PKM2 protein levels (also its RNA), particularly at the nucleus of both A549 and H1299 cells. Then, considering the information reported by Ferreira et al. (2019a), we proposed that, also in the nucleus of these human cells, *FA-SAT* ncRNA and PKM2 should interact to originate a ribonucleoprotein. Furthermore, once PKM2 was previously linked to the regulation of the cell cycle (Goldberg and Sharp 2012; reviewed in Tamada et al. 2012; Ao et al. 2017), we anticipated the fundamental role of the ribonucleoprotein complex in determining the outcomes described below.

The phenotypes resulting from the ncRNA depletion (with consequent disruption of the *FA-SAT* ncRNA/PKM2 complex) were also explored in terms of the cell-cycle progression of the A549 and H1299 transfected cells. Firstly, the impairment of the cell cycle was noticed, when this satDNA was silenced (the cells did not multiply over time as it happens in the controls), and it was accompanied by *MYC* downregulation, in both cell lines. In different works, the regulation of cell proliferation had already been linked to the transcriptional activity of *FA-SAT*, in cat and other human cells (Ferreira et al. 2019a), and of some other satncRNAs, for example the human satellite II (Bersani et al. 2015; Nogalski and Shenk 2020). The *FA-SAT* ncRNA/PKM2 disruption still resulted in increased cell death, specifically apoptosis, in both P53 WT and null transfected cells. Also in this situation, there was previous evidence of the contribution of the satncRNAs to the induction of phenotypes such as cell-cycle arrest/apoptosis (Goenka et al. 2016; McNulty et al. 2017).

In brief, with these results, we attested the *FA-SAT* ncRNA/PKM2 association in the A549 and H1299 human cells and its importance to the regulation of the cross-talk between the mitogenic and apoptotic pathways in them, highlighting the similarity of the results obtained for both cell lines under analysis.

IV.4. The P53 status does not affect the *FA-SAT* ncRNA/PKM2 disruption outcomes

As previously mentioned, in Chapter III, the use of both P53 WT and null human cells had as its main purpose to let us draw conclusions about the influence of the P53 status (presence or absence of this protein in the cells) in the *FA-SAT* silencing outcomes. Since similar cellular

and molecular (PKM2 and MYC) phenotypes were observed in both cell lines, we initially indicated that these processes should occur independently of the P53 status. To confirm this premise, we assessed the phospho-P53 (ser 15) levels in the *FA-SAT* silenced cells, once the phosphorylation at this residue is fundamental for the protein activation and function in cell-cycle arrest and/or apoptosis (Shieh et al. 1997; Loughery et al. 2014; Yang et al. 2019). The P53 WT cells showed an accumulation of phospho-P53 (ser 15), when the apoptosis was triggered by the depletion of *FA-SAT* ncRNA, suggesting the implication of this protein in mediating/triggering this outcome. On the other hand, in the P53 null cells, the phospho-P53 (ser 15) absence was confirmed, but the apoptosis is still the phenotype observed in the *FA-SAT* depleted cells. Then, there should exist some alternative cell pathways, responsible for the apoptosis activation in these cells. Some hypotheses may include the ERK activation and Akt inactivation (with downregulation of the Bcl-2 and Bcl-XL anti-apoptotic proteins), as reported by Chang et al. (2004), or even the p38-MAPK inactivation (with upregulation of the CDK inhibitor $p21^{Waf1/Cip1}$ and G1 cell-cycle arrest), as stated by Yu et al. (2014), for other cell death-promoting molecules/agents.

Therefore, we hypothesized that these phenotypes may be mediated by P53 or occur through alternative pathways (in its absence), noting that the final outcomes, i.e., inhibition of cell proliferation and apoptosis induction, will be independent of the P53 status.

In summary, with this work, we were able to determine/establish: 1) the *FA-SAT* ncRNA cellular profile, for the first time, in humans, highlighting the conservation of its nuclear location (in cat and human cells) and the presence of its transcripts along all the cell cycle of HeLa cells (with accumulation in the cell-cycle entry); 2) the importance of the *FA-SAT* ncRNA/PKM2 association for the regulation of the mitogenic and apoptotic pathways in human cells; 3) the independence from the P53 status of the switch between cell proliferation and apoptosis, promoted by the *FA-SAT* ncRNA/PKM2 disruption. Then, if hypothetically this complex was used as a target to control the cell-cycle progression in tumor cells, the absence of P53 (very common feature in cancer) would not compromise the apoptosis induction promoted by the *FA-SAT* silencing, adding a lot of potential and scope of action to this approach. Finally, our research has contributed to highlighting the importance of satDNAs and repetitive DNA sequences in our genomes, as functional elements capable of regulating various processes underlying cell life and whose dysregulation may be associated with various pathologies/diseases.

IV.5. Future perspectives

Despite the advances in knowledge achieved with this work, the study of *FA-SAT* and its ncRNA in humans should be deepened to better understand its cellular behavior and its role(s) in cell function and diseases, such as cancer. In this sense, the following steps must be taken and explored in the future:

- To perform the physical mapping of *FA-SAT* in the human chromosomes, combining DNA-FISH (with signal amplification systems, TSA) and bioinformatic approaches/tools;
- To detail the *FA-SAT* transcriptional profile here reported, carrying out studies on the ncRNA cellular dynamics (live-cell analysis) and its molecular characterization (transcripts size, full sequence and presence of polyA tail and/or 5'cap);
- To characterize similarly the *FA-SAT* ncRNA profile in human non-tumor cells, so we can compare with the data described in this work and infer about the state of (dys)regulation of its expression in cancer vs. normal cells;
- To explore the *FA-SAT* ncRNA/PKM2 interaction, performing co-localization and interatomic assays and evaluating the *FA-SAT* ncRNA levels in the PKM2-depleted cells (PKM2 siRNA);
- To identify the RNAs and proteins from mitogenic and apoptotic pathways (e.g., proto-oncogenes, tumor suppressor genes and anti or pro-apoptotic genes) that are up- or down-regulated in the *FA-SAT* ncRNA/PKM2 depleted cells (both in the P53 WT and null cells);
- To perform *FA-SAT* and P53 co-silencing assays using P53 WT (A549) cells, to mimic the P53 null (H1299) cells and compare the results from each cell line.

The information provided by these studies, together with the data here reported, will enable us to fully understand how the *FA-SAT* transcriptional activity can modulate specific cellular mechanisms (namely, in the cross-talk between the mitogenic and apoptotic pathways, by the *FA-SAT* ncRNA/PKM2 complex) and, therefore, to determine the reason why this satDNA is so highly conserved in our genomes, evidencing its importance in them.

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