Epigenetics and Its Applications to the Progression Model of Pancreatic Cancer

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Abstract

Defined as heritable changes in gene expression, which are not due to any alteration in the DNA sequence, epigenetic pathways have come to the forefront of research in disease, and in particular, cancer. In fact, these pathways are more prevalently altered in cancer than genetic alterations and most important, can be reversible, lending themselves as attractive therapeutic targets. This chapter will cover the basic aspects of transcriptional gene regulation, epigenetics, and chromatin dynamics and then focus on the intricacies of its application to pancreatic cancer biology and potential therapeutics. In addition, a model for better understanding pancreatic cancer is outlined to expand the highly provocative and productive "mutation centric" progression model, as defined by Hruban and colleagues, into a current model that formally includes chromatin-induced and noncoding RNA-induced epigenetic changes, as well as other alterations that result from changes in nuclear shape. This model offers a compass for further considerations aimed at illuminating the field of pancreatic cancer biology, diagnosis, therapeutics, and chemoprevention, in a similar, prolific manner as the original model.

Keywords

Epigenetics · Transcription · Chromatin dynamics · DNA methylation · Histone · Non-coding RNAs · Nucleus · Nuclear shape · Pancreatic cancer

Introduction

The phenomenon of epigenetics involves the regulation of gene expression via chromatin modifications and remodeling. Interestingly, an embryo is defined as human by the amount and sequence of DNA, which result from the fusion of the two parental gametes. However, as the embryo grows, cells will begin to differentiate from each other with this same amount and sequence of DNA. The ultimate results of the differentiation process seen in a young adult clearly show that despite all cells within the same organism carrying the same DNA sequence, a neuron, for instance, is totally different than a pancreatic acinar cell. Meditating on this phenomenon can leave one breathless. If one supposes that these two cells are independent unicellular organisms instead of both originating from a human, it would not be apparent that they have the same genome. Epigenetic mechanisms are responsible for defining cell phenotypes during the differentiation process by modulating the expression of the same genome in a different manner that is inheritable in each somatic cell division. Therefore, this chapter will (1) review the basic aspects of molecular mechanisms that are important for understanding gene regulation and epigenetics; (2) discuss the current model for better understanding pancreatic cancer,

which expands the extremely provocative and productive "mutation centric" model defined by Hruban et al. in 2000 [1] into one that formally includes chromatin-induced and noncoding RNA-induced epigenetic changes, as well as other modifications that result from alterations in nuclear shape; and (3) briefly consider drugs that may be important for the chemoprevention and/or treatment of pancreatic cancer.

Basic Concepts in Epigenetics

The study of epigenetics has been an example of how applicable the epistemological concepts behind the Thomas Kuhn's seminal work, "The Structure of Scientific Revolutions," are to this science [2]. In this work, Kuhn proposes that science moves ahead not by the incessant generation of data, but by work that changes preexistent paradigms. This is sometimes referred to as an epistemological fracture, meaning that the conceptual framework that was valid yesterday has evolved into a new theoretical framework that better explains reality. Therefore, the basis of epigenetics will be discussed through the progression of paradigms that have dominated this science at different stages of its development until today. These basic paradigms should be integrated into a picture of how chromatin and the transcriptional regulatory machinery work together in order to mediate epigenetic inheritance in somatic cells.

Evolving Paradigms in the Field of Transcription, Chromatin, and Epigenetics

The Universality of Promoters

This is the story of a remarkable journey since the work of Jacob and Monod [3] to the large amount of work that went into discovering the transcriptional mechanisms that regulate basal levels of expression before either activation or repression can occur (Basal Transcription). Prokaryote cells have only one RNA polymerase that binds to the promoter of genes and, aided by a transcription factor (factor σ), initiates the synthesis of an RNA molecule (Transcription) (reviewed in [4]). A remarkable finding is that promoters from bacteria to human contain similar sequences (e.g., TATA box). This concept has supported the prediction that the regulation of gene expression throughout evolution has been mechanistically very similar. This level of similarity was remarkable in its time, but was distant from the entire actuality. Hardcore evidence for the functional evolutionary-conservation thinkers has been further supported by the discovery that, at the atomic resolution, the tridimensional structure among RNA polymerases is strikingly high [5]. Thus, this theoretical framework paved the way for the search of eukaryote molecules that mediate transcription.

The RNA Pol II Components and the General Transcription Factors

The discovery of an RNA polymerase from eukaryotic cells highly stimulated studies aimed toward understanding transcriptional regulation [6]. However, the complexity of eukaryotes became apparent in comparison to bacteria, in particular, with the isolation of two additional RNA polymerases from higher organisms for a total of three RNA polymerase molecules, referred to as RNA polymerase I, RNA Polymerase II, and RNA polymerase III (reviewed in [7]). The intricacies of the eukaryotic system became further evident upon attempts to reconstitute transcription from isolated RNA polymerase II complexes bound to the core promoter of genes involved in basal transcription [8]. Transcription initiation at RNA polymerase II promoters in eukaryotes, which is the focus of the current chapter due to its association with protein-encoding gene expression, involves the assembly of a megadalton, multiprotein complex, comprised of the polymerase itself, as well as a variety of associated factors, known as the General Transcription Factors (GTFs). These general transcription factors function to properly position RNA pol II on the promoter DNA and to interact with transcriptional activators. The isolation and reconstitution of transcription in vitro to derive the resultant theoretical framework required several decades, until the details of the paradigm described in the following paragraph emerged.

The Step-Wise Assembly of the RNA Pol II Complex Versus the Holoenzyme Complex

To focus on the process of transcriptional initiation, it is most logical to begin with a description of RNA polymerase II complex, the transcriptional enzyme complex, responsible for making the protein-encoding RNA molecules, which includes the general transcription factors. Two paradigms exist for initiation of promoter occupancy by the RNA pol II complex: individual general transcription factors and the enzyme may be assembled in situ on the promoter in a step-wise fashion or the entire machinery and its associated factors bind the promoter collectively as the pre-assembled polymerase II holoenzyme (reviewed in [9]). Based on the step-wise assembly paradigm, the eukaryotic core promoter serves as a platform for the assembly of the transcription preinitiation complex (PIC). PIC assembly commences with TFIID binding to the TATA box, initiator, and/or downstream promoter element (DPE) present in most core promoters. The concept of the PIC was originated primarily from results of in vitro reconstitution assays, which subsequently led to the isolation of the GTFs that enter into the process of transcription in a step-wise manner to aid RNA polymerase II. These proteins include, in order of association to the promoter, TFIID, TFIIB, TFIIA, TFIIF, TFIIE, and TFIIH (reviewed in [10]). TFIID, the initial GTF to bind for PIC formation, is the only GTF with site-specific DNA binding ability and in itself a complex containing the TATA-binding protein (TBP) and numerous TBP-associated factors, termed TAF_{II}s. Subsequently, TFIIB recognizes the TFIID-promoter complex and, along with TFIIA, stabilizes the

nucleoprotein complex, which allows TFIIF to escort RNA pol II to the promoter. The interaction between TFIIB and RNA pol II is crucial for defining the proper start site of transcription [11]. Once RNA pol II is stably positioned, it is unable to initiate RNA transcription until the recruitment of two additional GTFs, TFIIE, and TFIIH. Transcriptional initiation requires two functions of the TFIIH, a helicase activity to open the double stranded DNA since the RNA polymerase will copy only a single strand of a gene, and a CDK kinase activity, which hyperphosphorylates the tail of the RNA pol II molecule to initiate transcription.

Two major discoveries have been the existence of the Mediator Complex [12], which is necessary for full function of the RNA pol II, as well as the possibility that the RNA pol II enzyme, GTFs, and Mediator could be preassembled to form the RNA polymerase II holoenzyme (enzyme with all the parts) prior to promoter recruitment. This process forms the basis of the holoenzyme paradigm [9]. The knowledge derived from both the step-wise assembly and the holoenzyme paradigm is currently operational.

The Promoter-Bashing Paradigm, Cis-Regulatory Sequences, and Sequence-Specific Transcription Factors

At the same time experiments were actively underway to understand the mechanisms regulating basal transcription, other investigators were searching for the basis of regulated transcription, namely, transcriptional activation (gene induction) and/or transcriptional repression (gene silencing). For this purpose, investigators adopted concepts and tools to dissect this process, including fusing promoter regions to reporter genes and performing deletions and site-directed mutagenesis for teasing out potential sites that could bind sequence-specific transcriptional regulators, which provided fruitful information as the promoter-bashing paradigm. In addition, promoter footprinting and Electrophoretic Mobility Shift Assays (EMSAs) were utilized to determine transcription factor binding to specific DNA sequences, called cis-regulatory sites [13]. These factors act either as monomers, such as the pancreatic tumor suppressor, and sequence-specific transcription factor, KLF11 [14], or as a complex, such as PTF1 [15], which recognizes the promoters of many acinar cell genes in a trimeric homeodomain complex including P48 and HEB. Some of this knowledge not only advanced the concept of transcription, but also generated useful tools for the Pancreatology field, since several tissue-enriched or developmental time-specific promoters (reviewed in [16]) are the key requirement for the creation of several animal models for pancreatitis and cancer.

The Coactivator-Corepressor Hypothesis

Studies designed to better decipher the way that sequence-specific transcription factors regulate gene expression led to the concept that these proteins behave as adaptors between the DNA and proteins that either induce or impede RNA pol II

transcription. This concept was based upon the recognition this type of transcription factor was modular in structure, composed of a DNA binding domain and a transcriptional regulatory domain to influence the rate of mRNA synthesis (reviewed in [17–19]). Conceptually, proteins responsible for promoting activation were called coactivators, while any corresponding repressor proteins were termed corepressors. Initially, some investigators searched for these factors among the hundreds of proteins that form the RNA Pol II holoenzyme. Indeed, interactions of transcription factors with certain members of the holoenzyme were necessary for regulated transcription. However, at the same time, a new era in studying the role of chromatin proteins was being born and starting to dominate, at the mechanistic level, the field of gene expression and apoptosis, proliferation, senescence, stem cell biology, cell migration, oncogenesis, tumor suppression, DNA replication, DNA repair, ploidy, as well as other processes integrally associated with the development and maintenance of the pancreatic cancer phenotype. For instance, it is now known that histone deacetylases (HDACs) play significant regulatory roles in gene expression during cancer [20], in particular in silencing tumor suppressor genes, and select inhibitors of these proteins are approved for clinical use in lymphoma and multiple myeloma and others are in various phases of clinical trials for the treatment of diverse malignancies [21]. HDACs are recruited into different protein corepressor complexes, which are brought to promoters via the transcriptional regulatory domain of a distinct transcription factor bound to DNA (reviewed in [22]). As a result, this transcription factor effectively deacetylates histones, which serves as a signal for gene silencing (Fig. 1). The reversal of this state is achieved through the function of histone acetylases enzymes (HATs), such as CREB binding protein (CBP)/P300 and P300/CBP-Associated Factor (PCAF) (reviewed in [23]). The deregulation of these types of enzymes leads to the aberrant activation of oncogenes (Fig. 2). Other nonhistone chromatin proteins function either as coactivators or corepressors via distinct mechanisms, as mediators of histone methylation, ubiquitination, sumoylation, and other modifications, which inform the cell toward dynamically changing gene expression patterns according to the corresponding function.

Chromatin Dynamics Forms the Basis of Epigenetics

Work on the role of histones in nuclear cell biology was very active in the 1970s with a detailed analysis of nucleosome composition and DNA packaging [24]. In terms of transcription, histones and nucleosomes were believed to be rich solely in heterochromatin, which is transcriptionally silent, and relatively poor in euchromatin, which is transcriptionally active. Unfortunately, however, how these states could be interchanged, meaning that chromatin was more dynamic than previously speculated, remained poorly understood until the 1980s and received a boost at the turn of the century (reviewed in [25]). Research on transcriptional regulation and its relevance to biological and pathobiological processes grew significantly with the discovery that indeed, chromatin is dynamic, often switching from euchromatin to heterochromatin and vice versa. Chromatin dynamics is regulated by (a) signaling

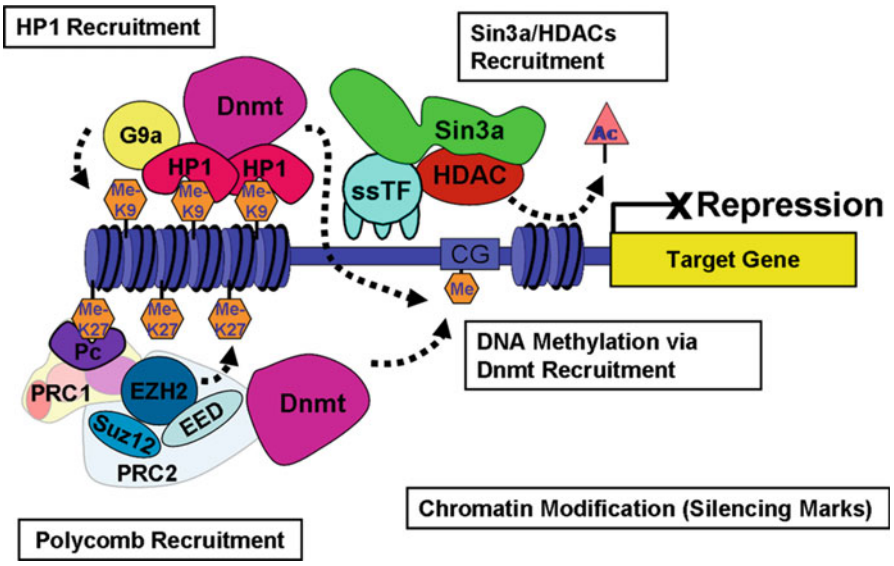

Fig. 1 Examples of Epigenetic-Mediated Tumor Suppressor Gene Silencing. This cartoon depicts a model for various roles of chromatin dynamics in tumor suppressor gene silencing, participating in the cancer phenotype. Several different mechanisms of epigenetic-mediated gene silencing can accomplish the same outcome of tumor suppressor gene silencing, including the HDAC system, polycomb proteins, and HP1 proteins. For example, a sequence-specific transcription factor (ssTF) may recruit the Sin3a-HDAC complex to a target gene promoter. The recruitment of Sin3a-HDAC to the promoter facilitates the remodeling of surrounding chromatin with silencing marks, namely the deacetylation of histones. Removal of acetylation signals short-term repression of a target gene and in addition, primes the histone for receiving additional long-term silencing marks, such as methylation of K9 or K27 on histone H3, binding marks for HP1 and polycomb, respectively. The recruitment of HP1 to a gene promoter facilitates the further recruitment of the G9a methylase, which creates more methyl-H3K9 silencing marks and thus, more HP1 binding sites. In addition, HP1 can recruit a DNA methyltransferase (Dnmt) to the promoter. In a similar manner for the polycomb group proteins, PRC1 recruitment results in the binding of the PRC2 complex, which contains the H3K27 methylase EZH2. The PRC2 complex also is capable of recruiting the DNA methyltransferases

events that form the basis of the histone code and subcodes, (b) mechanochemical enzymes that move nucleosomes from cis-regulatory sequences, an essential step in transcription, as well as (c) histone chaperones, which remove histones from nucleosomes to either activate or silence gene expression. Noteworthy, chromatin dynamics determines the epigenetic inheritance of a phenotypic trait either from the germ line (imprinting) or from one somatic cell to its daughter. DNA content is the same throughout the body, yet different types of cells with distinct characteristics and functions exist to create various organs and biological systems. Often not considered, the exact same DNA is in every cell, and thus, the distinction in the type of cell it becomes lies within epigenetics, and in particular, chromatin dynamics. Following, these three areas of chromatin dynamics are described in further detail.

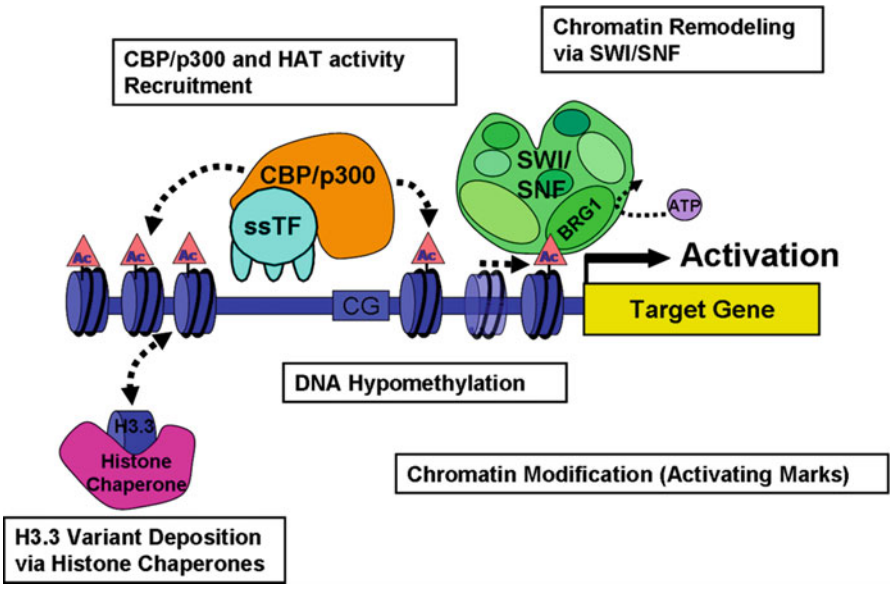

Fig. 2 Examples of Epigenetic-Mediated Oncogene Activation. This cartoon depicts a model for the role of chromatin dynamics in promoting the cancer phenotype through oncogene activation. In this model, a sequence-specific transcription factor (ssTF) triggers the recruitment of CBP/p300 (or PCAF) to a target gene promoter. The recruitment of CBP/p300 to the promoter also provides HAT activity, which facilitates the modification of surrounding histones to create "active" chromatin with acetylated histones. Addition of acetylated marks to histones signals activation of transcription through recruitment of other bromodomain-containing proteins, such as the SWI/SNF family of chromatin mechanochemical remodelers, which via the expenditure of ATP facilitate structural relaxation of chromatin and thus, access to transcriptional machinery. Additional players in the process of gene activation can include the histone chaperones, which through the exchange of histone variants, such as histone H3.3, provide activating signals. In addition, demethylation of DNA can trigger the activation of an oncogene promoter

The Histone Code and Subcode Hypotheses: Codifying Gene Activation and/or Silencing and Epigenetics

Elegant work from many laboratories around the world found its conceptual integration in the development of the histone code hypothesis [26]. Before describing this theoretical framework for understanding transcription and epigenetics, one should remember that histones are small, basic proteins that are extremely conserved throughout evolution [27]. To illustrate how conserved histones are and better explain how the histone code hypothesis operates, histone H3 (H3) is used here as an example, although the code considers all the histones and its genetic variants.

The first 24 amino acids of H3 are nearly identical in most organisms, known as the histone H3 tail. Collectively, the histone "tails" have been defined, from analysis of their crystal structure, as the regions of the histone sequences that extend from the nucleosomal disk [28]. The H3 tail contains several serine(S), threonine(T), and tyrosine(Y) residues, which have the ability to undergo phosphorylation, and other

residues, such as lysine(K) and arginine(R), which can be extensively modified by methylation, acetylation, ubiquitination, and sumoylation [26]. In fact, the lysines and arginines have the potential to possess different states of methylation, namely mono-, di-, and tri-methylated for lysines and mono-, symmetrically di-, and asymmetrically di-methylated for arginines [29]. These histone modifications have come to be known as "marks" because in many cases, they are utilized as clues for epigenetics. For instance, the Polycomb complex, which keeps stem cells in their undifferentiated state, binds to trimethylated K27 of H3 in order to mediate heterochromatin formation on target promoters and, as consequence of this event, to facilitate gene silencing [30]. This is one of the mechanisms for epigenetic inheritance in human somatic cells where the K27 trimethyl mark must be removed to initiate the hierarchical cascade of gene expression that leads to a cell fate decision. Interestingly, as described below, this epigenetic mechanism is often used for permanently silencing tumor suppressors without the need of gene mutation or deletion (Fig. 1). A similar function in gene silencing is performed by another protein, HP1, which binds to di- and tri-methylated K9 of H3. The histone code hypothesis predicts that the type, location, and combination of histone marks determine whether a gene is expressed or silent under a particular set of circumstances. Using HP1 as a model of a histone mark-binding protein, these nonhistone proteins were found to also be modified by the same enzymes that are responsible of creating the histone code, appearing to act in the fine-tuning of the instructions given by the histone marks [31], which has been subsequently supported by additional modifications in HP1 and other epigenetic regulators [32–34]. For instance, a required step for entering into cell senescence is the phosphorylation of HP1γ at residue S83 (S93 from alternative start site) [35], suggesting that this modification instructs HP1 to regulate the gene expression of key genes which will epigenetically influence the cell into senescence. In fact, the underlying mechanism driving these subcodes is believed to be "histone mimicry," which is the presence of histone-like modification cassettes within nonhistone proteins [36]. Thus, the histone code and its subcodes have fueled a new era of great productivity and optimism in the field of transcription, chromatin dynamics, and epigenetics, in particular as it relates to cancer.

Nucleosome Remodeling Machines

Nucleosome remodeling machines, containing ATP-dependent mechanochemical activity (molecular motors), were discovered using biochemical methods and in vitro assays. Using these approaches, numerous laboratories have isolated protein complexes that move nucleosomes along DNA thereby removing a repressive effect of histones on a specific cis-regulatory sequence. These nucleosome remodeling complexes include SWI/SNF, NuRD (nucleosome remodeling and deacetylation), and CHRAC (chromatin accessibility complex) (reviewed in [37]). Several of these molecular machines are conserved from organisms ranging from yeast to human. To demonstrate the basic mechanisms of these nucleosome remodelers, the SWI/SNF complex will be used as an example, which is the human homolog to the *Drosophila* trithorax complex [38]. The function of complexes like SWI/SNF is essential for the

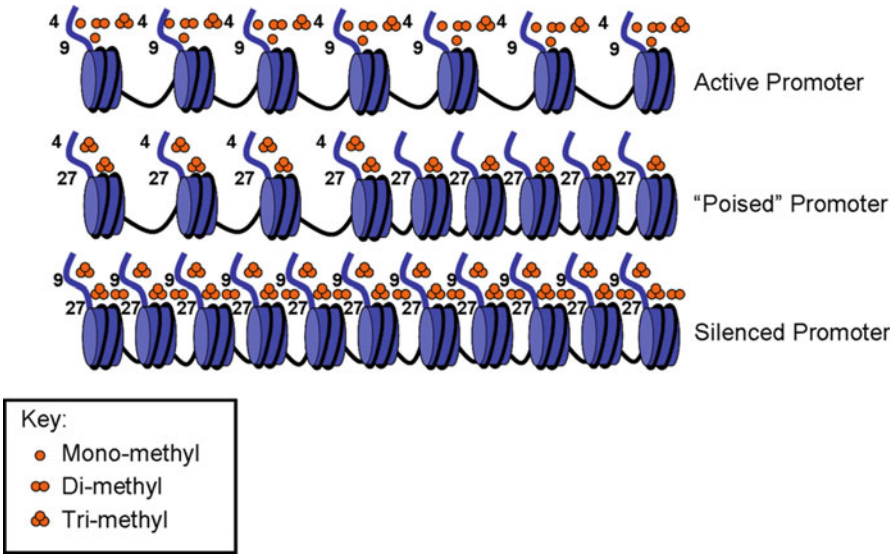

Fig. 3 Dynamics of Chromatin Marks on Promoters. The figure demonstrates three different promoter states of chromatin marks: active, "poised," and silenced (adapted from [44]). Nucleosomes encompassing the promoter region of a gene are shown. The numbers indicate the corresponding amino acid of the histone H3 tail. The orange circles represent the degree of methylation with multiple states possible for a given signal. For example, on active promoters, the chromatin marks are a signal of gene transcription, such as mono-, di-, or tri-methylation of K4 of H3 and mono-methylation of H3K9. Active promoters are also enriched in H3, H4, and H2A acetylation (not shown). On a "poised" promoter, a combination of active and repressive marks can leave genes ready for activation and forms a "bivalent domain." The promoter regions of this type are enriched in the repressive trimethyl-H3K27 mark, whereas the region around the transcription start is also enriched in the active trimethyl-H3K4 mark. Finally, a silenced promoter contains inactive chromatin marks. These nucleosomes are enriched in H3K9 tri-methylation (and sometimes di-methylation, not shown) and H3K27 di- and tri-methylation

expression of a myriad of genes via its recruitment to chromatin, hydrolysis of ATP, and utilization of this energy to remodel nucleosomes (Fig. 2). While *Drosophila* only possesses a single Swi2/Snf2 complex with ATPase activity, called Brahma (Brm) [39], mammals have two homologues, BRM and BRG1 [40]. The amino acid sequences of these two are 75% identical with broad expression. However, these subunits are mutually exclusive, since a single SWI/SNF complex contains either BRM or BRG1. Thus, there are several subtypes of SWI/SNF complexes that can be divided based on the ATPase molecule that generates the mechanochemical force for nucleosome movement. Interestingly, the genes encoding these subunits have been found to have mutations and/or loss of expression in some human tumor cell lines, as well as primary tumors, including pancreatic cancer [41, 42].

The trithorax complex recognizes methylated H3K4, actively participating in the epigenetics and chromatin dynamics of the cell. For instance, stem cells are characterized by having a subset of genes with dual marks, methylated at both H3K4 and

H3K27 (Fig. 3). These gene promoters are known to be in a "poised" state, since they are repressed by polycomb in the stem cells, but after removal of the dominant H3K27 mark, the remaining methylated H3K4 will signal for activation, leading to the initiation of cell differentiation [43]. Therefore, although heterochromatin is repressive, nucleosome remodeling machines, by binding to specific histone marks, sometimes already present on a promoter along with the silencing mark, will convert the region into active euchromatin. Tumorigenesis exhibits the culmination of alterations in several genetic pathways. Therefore, as is the case with many of the global epigenetic effects discussed in this chapter, it would only take a single mutation to inactivate a large subset of SWI/SNF complexes (such as a BRG1 mutation) to perturb the regulation of numerous downstream genetic pathways and as a result, trigger robust growth-promoting effects (Fig. 2).

Histone Chaperones

The discovery of histone chaperones constitutes later developments within the area of transcription [44]. The search for this type of proteins initiated from the understanding that there were many histones and histone variants that could occupy a nucleosome. For instance, histone H3 has four main isoforms in mammals [45]. Some of these variants act as activators, while others act as repressors in the context of a nucleosome [46]. Deposition of histone variant H3.3 has been associated with transcriptionally active genes in plants, flies, and humans. In addition to the possibility of different histone variants occupying a nucleosome, these variants are also substrates of enzymes that create histone marks. Therefore, the combinatorial effect between the existence of the histone variants and their participation in the histone code, which is known as the histone "barcode" [47], creates the possibilities of regulating activation or repression significantly complex. An important contribution to the field was the discovery that some histone variants are rapidly exchanged from nucleosomes, leading to the finding that this nucleosome-histone exchange codifies for either gene activation or silencing. Therefore, histone chaperones cooperate with the histone code in instructing cells to regulate a particular program of gene expression (Fig. 2). The role of histone chaperones involves binding highly basic histone proteins, which protects them from nonspecific interactions to facilitate either their deposition onto or eviction from DNA. Interestingly, despite their common functions, histone chaperone proteins structurally demonstrate highly divergent molecular structures and modest commonalities in their folds [47]. However, according to sequence-based predictions, these proteins have recently been shown to contain critical intrinsically disordered regions (IDRs) and acidic stretches, which are thought to play key roles in histone chaperone function, although this remains a currently active area of research.

Nuclear Shape and Nuclear Domains

The influence of nuclear shape in determining the tridimensional location of a particular gene within the nucleus in interphase is well known (chromosome

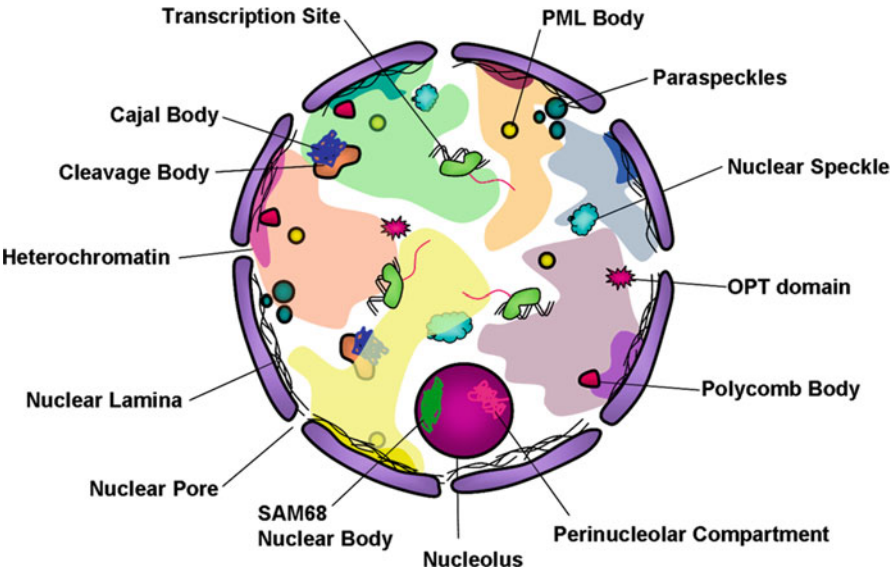

Fig. 4 Chromosomal Territories and Nuclear Domains. This cartoon of a mammalian nucleus illustrates the chromosomal territories and various nuclear bodies. Chromosomes occupy discrete territories in the nucleus. In addition, various functions within the nucleus occur in distinct locations, considered nuclear bodies or domains. Recent important and elegant work has demonstrated that alterations in nuclear shape will impact on these nuclear territories and domains, affecting gene expression in a manner resembles aging, polyploidy, and aneuploidy, all changes that are found in pancreatic cancer. Therefore, extending this area of research is of paramount importance for this field

territory) [48]. In addition, the nucleus consists of distinct nuclear domains with various components, which suggests that various nuclear functions occur at precise locations within the nucleus (Fig. 4). This knowledge supports the notion that changes in nuclear shape, by altering the nuclear position of the gene, can alter chromatin dynamics leading to aberrant gene expression. Clear support for this concept came from a naturally occurring mutation in the Lamin A gene [49]. Lamins are proteins that form intermediate filaments, which create a nuclear lamina covering the nucleus and extend toward the interior of this organelle to form a skeleton (reviewed in [50]). Thermodynamically speaking, the efficiency of an enzyme is better when in association with a surface rather than free floating in solution. Therefore, this lamin-based skeleton is necessary for all the processes that occur in the nucleus by helping to compartmentalize and concentrate specific molecular machineries into nuclear domains, which can be considered the nuclear equivalent of the cytoplasmic organelle, though not surrounded by a membrane. Mutations in lamin A significantly change nuclear shape, generating a new pattern of gene expression, which is responsible for the phenotype of premature aging and cancer in the Hutchinson–Gilford progeria syndrome [49]. With increasing focus on the functional relevance of morphological changes in the size and shape of the nucleus

during tumorigenesis, studies have found both increased and decreased lamin A/C levels to be correlated with poor prognosis in human cancers [51]. Notably, in considering the critical role of the tumor microenvironment in pancreatic cancer, aberrant levels of lamin A/C are also associated to collagen deposition and fibrosis, suggesting its effect reaches beyond the nuclear structure to influence the tissue architecture and microenvironment. This has inspired our laboratory to predict that some of the gross nuclear changes observed early during the progression of histopathological lesions in pancreatic cancer are not a consequence of cancer, but rather these changes help in the development and/or maintenance of this malignant phenotype. Therefore, nuclear shape must be included as a candidate modifier of pancreatic cancer progression, since the transition of PanIN 1B to PanIN 2 requires changes in nuclear shape [52]. The hypothesis is that these nuclear changes are responsible for extensively altering gene expression, independently of other epigenetic mechanisms, and thereby significantly contribute to the progression and maintenance of the pancreatic cancer phenotype. Thus, the "Triple Code Hypothesis," as illustrated in Fig.5a, is an integration of changes in DNA, such as mutation or deletion, which are an established part of cancer progression, alterations in chromatin, which are increasingly recognized as well, and the addition layer of changes in nuclear structure [53].

Epigenetics: Developing a Novel and Comprehensive Genomic-Epigenomic Model for Pancreatic Cancer that Includes Chromatin Dynamics and Nuclear Shape

The revolution of somatic genetics in the field of cancer brought about by the model developed by Fearon and Vogelstein in colon [54], which later led to an adaptation to the pancreas by Hruban et al. [1], opened a fruitful era for pancreatic cancer research, spanning approximately two decades. The basic premise of somatic genetics in cancer is that if a gene, which is suspected to play a role related to cancer, is overamplified, for instance, Myc in brain, it behaves as an oncogene, but if it is downregulated, like p16 in pancreatic cancer, it behaves as a tumor suppressor. Due to this premise, in the pancreatic cancer field, the changes in expression of both oncogenes and tumor suppressors, according to the Hruban model, were originally believed to occur via mutation or deletion and later with the work of Goggins, by promoter methylation [55–57]. The validity of this model has been elegantly demonstrated using Genetically Engineered Models (GEM), primarily supported by NIH via the "Mouse Model Consortium" funded by NCI [58].

In addition to the recognition of the outstanding contribution, this progression model of somatic genetics has had in advancing cancer research, the revised progression model for pancreatic cancer also must take into consideration the theoretical framework of epigenetics, and specifically, changes that occur at the protein level in the absence of DNA changes, such as deletion, mutation, or even promoter methylation. For instance, upon reading through the Hruban model of pancreatic cancer, in which the underlying conceptual framework is genetic in nature, one can infer that

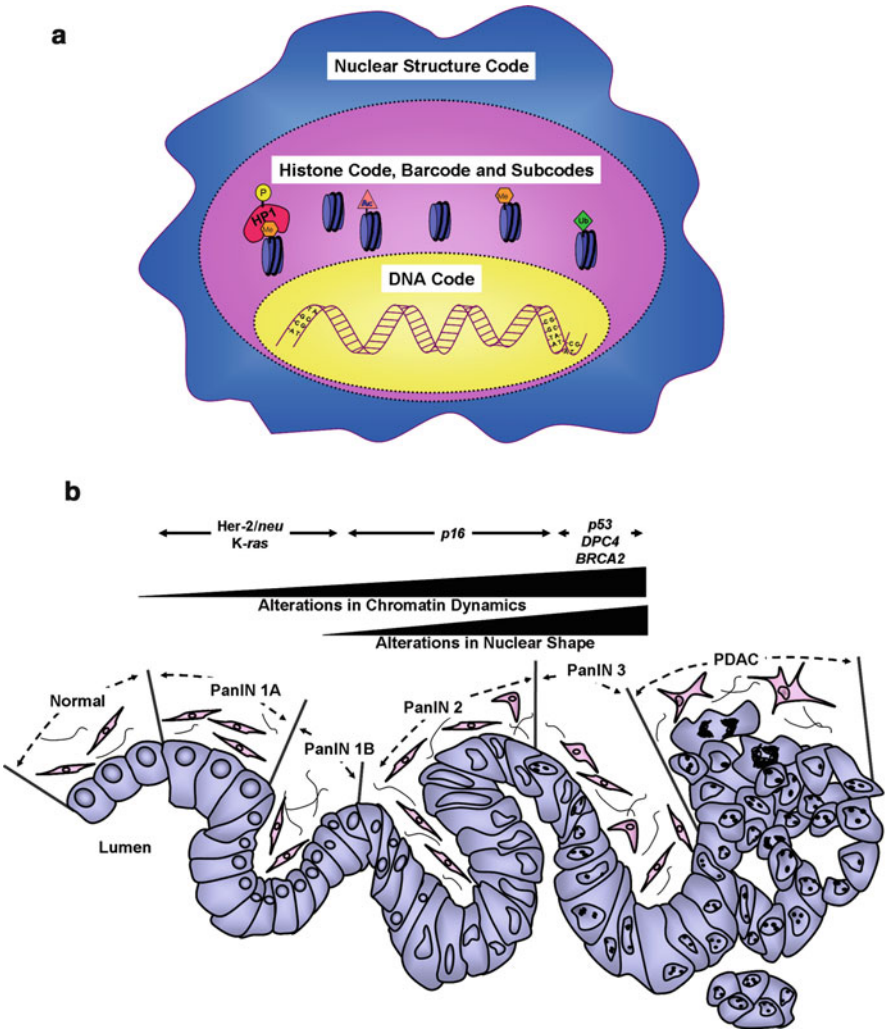

Fig. 5 (**a**) **The Triple Code Hypothesis**. This figure summarizes the integration of the well-known DNA-centric hypothesis for the establishment and maintenance of the cancer phenotype, which includes mutations and deletions, with changes in chromatin, signaled through the histone code, barcode, and its subcodes, and alterations in nuclear structure to form the "Triple Code Hypothesis." This "Triple Code Hypothesis" has formed the basis of the more comprehensive progression model for pancreatic cancer, proposed in **b**. (**b**) **Revised Comprehensive Progression Model for Pancreatic Cancer**. The model developed by Hruban and colleagues [1] was fundamental for expanding the work of many laboratories in the area of somatic genetics in pancreatic cancer to allow better understanding of the relationship between the morphological progression and mutations/deletion of important oncogenes and tumor suppressor pathways. However, the model excludes emerging knowledge on critical steps that occur between these mutations and even the potential cause of subsequent mutations and deletions. Most of these changes are epigenetic in nature with the underlying basic mechanisms of both chromatin dynamics and nuclear shape. Thus, a revised model for the progression of pancreatic cancer [53], which not only incorporates the

pancreatic cancer progresses through multistep mechanisms with different lesions evolving via mutations in different genes. However, this model does not explain what protein-mediated epigenetic changes, which can take place between the occurrences of landmark mutations, are responsible for cancer progression, nor this model has proven that a later mutation is caused by an earlier one. Therefore, in the following paragraphs, examples of epigenetic changes that occur in time between mutations and can lead to tumor suppressor silencing are provided, starting with DNA methylation and proceeding through some modifiers of chromatin. These examples highlight a paradigm for the progression of pancreatic cancer, which includes two additional types of phenomena (besides genetics), namely changes in chromatin dynamics and nuclear shape (Fig. 5b). The hope is for new investigators in this field to dive into pancreatic cancer with a more in depth mechanistic approach than using only the tools of molecular pathology and a combination of a multitude of arrays for different purposes.

While the field of epigenetics is vast and includes mechanisms of gene activation and repression, this chapter will focus on changes in epigenetics and chromatin dynamics that can silence tumor suppressor genes via mechanisms that are totally independent of either genetic deletions or mutations. In fact, in the case of *p16*, which is utilized here as a prime example for pancreatic cancer in the following paragraphs, epigenetic mechanisms lead to the final methylation of this gene, which should take the readers to consider that chromatin changes can occur before and lead to the inactivation of landmark mutations that were described in the original paradigm. Therefore, this journey will begin with a brief description of this final read-out in epigenetics, DNA methylation, since it is the most commonly known epigenetic alteration, and continue temporally backwards in epigenetics toward changes in chromatin and their modifiers. In addition, studies in the epigenetics of noncoding RNAs in pancreatic cancer will be described, which is the most recent area to develop in the field.

DNA Methylation

As mentioned, DNA methylation was the first type of epigenetic change to be studied as a mechanism for the inactivation of tumor suppressors [59]. DNA methylation occurs on dinucleotide CpGs, where cytosines precede guanines. The process of DNA methylation entails the addition of a methyl group to the number 5 carbon of

Fig. 5 (continued) elegant and extremely important data generated under the premise of the original model but, in addition, formally includes chromatin-induced and noncoding RNA-induced epigenetic changes, as well as other alterations caused by changes in nuclear shape, is illustrated. This model will hopefully serve as a compass to guide future experiments in these underexplored and yet crucial areas of knowledge. Experiments aimed at addressing the contribution of these phenomena to pancreatic cancer progression and their potential translation to clinical applications will be among the most promising areas of our field

the cytosine pyrimidine ring, which ultimately silences gene expression. Noteworthy, DNA methylation normally has significant physiological significance, as with genomic imprinting to ensure monoallelic expression and hypermethylation of repetitive genomic sequences to prevent chromosomal instability, translocations, and gene disruption caused by the reactivation of transposable DNA sequences. However, during tumorigenesis, aberrant DNA methylation can assist the cancer phenotype.

In pancreatic cancer, DNA methylation has been known for a long time as a mechanism to inactivate tumor suppressor genes, such as well-known inactivation of the p16 promoter via methylation [60]. In addition, loss of methylation of a normally silenced promoter in pancreatic cells, such as the gene encoding the hematopoietic-specific guanine nucleotide exchange factor, *VAV1*, can lead to its misexpression [61]. Initial methodologies only provided insights at the single gene level, but fortunately, recent developments in methodologies have advanced enough to perform genome-wide scale gene methylation analysis. With validity to both methodologies, methylation analysis of a single gene is practical as a specific candidate gene approach, while the genome-wide analysis possesses power in its unbiased approach. Several techniques utilized for methylation analysis include methylation-specific PCR, sequencing after bisulfite treatment, as well as mass spectrometry.

Although individual genes were discovered to be methylated in advanced pancreatic cancer, current evidence supports the idea that aberrant methylation occurs very early during the histopathological progression of this neoplasia. Using a specific gene candidate approach, Rosty and colleagues demonstrated that PanIN lesions in patients with chronic pancreatitis show loss of *p16* expression [62], suggesting that this alteration may contribute to the predisposition of patients with chronic pancreatitis to develop pancreatic ductal adenocarcinoma. Interestingly, in a study involving large-scale methylation analysis with subsequent confirmation via methylation-specific PCR, Sato and colleagues analyzed DNA samples from 65 PanIN lesions for methylation status of eight genes identified prior by a larger scale microarray approach as aberrantly hypermethylated in invasive pancreatic cancer [63]. Of the PanIN lesions examined in this study, methylation at any of these genes was identified in 68% of samples. Even more importantly, in the earliest lesions, which are the PanIN-1A, aberrant methylation was present in approximately 70%. Among the genes analyzed, methylation prevalence increased from PanIN-1 to PanIN-2 for *NPTX2* and from PanIN-2 to PanIN-3 for *SARP2*, *Reprimo*, and *LHX1*. The most striking result from both studies is that aberrant CpG island hypermethylation begins in early stages of PanINs and its prevalence progressively increases during neoplastic progression.

Additional studies on methylation patterns in pancreatic cancer compared to nontumor pancreatic tissues have followed to demonstrate a high level of differently methylated regions (DMRs) between the two groups, which offer a large list of candidate genes to serve as diagnostic biomarkers or therapeutic targets [64, 65]. A more recent study, using reduced-representation bisulfite DNA sequencing (RRBS) followed by targeted methylation-specific PCR to validate novel DNA methylation markers strongly associated with pancreatic cancer, could discriminate pancreatic

cases from controls in pancreatic juice, which offers clinical significance in terms of detection and would benefit further validation in patients with early PanIN lesions [66]. With the current interest in circulating cell-free DNA (cfDNA), Henriksen and colleagues identified differences in cfDNA promoter hypermethylation between malignant and benign pancreatic disease to suggest its utility as a noninvasive, blood-based screening tool for pancreatic cancer [67]. Thus, aberrant DNA methylation not only continues to reconfirm its clear role in the progression of pancreatic cancer, but holds promise as a diagnostic marker. Furthermore, since the current evidence indicates that methylation occurs at an early preneoplastic stage, pharmacological agents that target methylation, which are discussed in a subsequent chapter on "Epigenetic Pharmacology," may be effective not only for treatment, but perhaps also for chemoprevention.

Histone Acetylation and Deacetylation

An important mechanism underlying the epigenetic regulation of gene expression is the acetylation and deacetylation of lysine residues within histone tails [68]. For acetylation, this process occurs via HATs, such as CBP, P300, and PCAF, to result in gene expression activation, whereas deacetylation is mediated by two different families of HDACs, resulting in gene silencing. Together, these enzymes provide a fine-tuned mechanism, which upon alteration has the possibility to cause the activation of oncogenic pathways (Fig. 2) and the silencing of tumor suppressors (Fig. 1). However, apart from other epigenetic regulators, such as the polycomb complexes and HP1, which are discussed below, HATs and HDACs mediate short-term responses, a fact that should be taken into consideration when thinking about these molecules as potential therapeutic targets in cancer [68, 69].

As discussed, transcriptional regulation is mediated by the DNA binding properties of sequence-specific transcription factors and the recruitment of trans-activators or repressors to ultimately cause effects that alter chromatin structure and dynamics. Studies have demonstrated that HDAC activity is increased in various tumors compared with normal tissue, and this increase in HDAC activity has been associated with transcriptional repression of tumor suppressor genes that cause growth inhibition and apoptosis [70]. In a study performed by Blasco and colleagues, the differential gene expression in a pancreatic cancer cell line upon induction of apoptosis was analyzed using cDNA arrays [71]. Among the genes differentially expressed, one that was studied for further validation was histone deacetylase 1 (HDAC1). Inhibition of HDAC activity led to an increase in the level of apoptosis, in parental cells and doxorubicin-resistant cells. Thus, this study suggested that HDAC1 could be a possible target to develop modulators in cancer chemotherapy that would increase or restore apoptosis. In another study performed by Ouaïssi et al., approximately 80% of pancreatic adenocarcinoma samples examined showed a significant increase of HDAC7 RNA and protein levels [72]. Interestingly, in contrast to the pancreatic adenocarcinoma samples, HDAC7 RNA levels were reduced in samples from chronic pancreatitis, serous cystadenoma, and intraductal

papillary mucinous tumor of the pancreas (IMPN), suggesting that increased expression of HDAC7 can discriminate pancreatic adenocarcinoma from other pancreatic types of tumors. Immunohistochemical assessment of HDAC1, HDAC2, HDAC4, and HDAC6 protein levels in 70 PDAC patient tissue samples demonstrated enhanced HDAC1 levels in association with increased tumor proliferative capacity, while elevated HDAC4 expression was significantly correlated with the absence of organ metastases [73]. Significantly longer survival times were noted in patients with high HDAC1 and HDAC6 levels compared to those with low expression of these molecules, whereas HDAC2 had no significant association with any of the clinicopathological parameters considered. In addition, it has been shown that HDAC1 mediates transcriptional repression of the TGFβRII promoter in pancreatic ductal adenocarcinoma cells via recruitment to a specific Sp1 site [74]. This Sp1 site can be occupied by TGFβ-inducible members of the KLF family, including KLF14 [75] and the pancreatic tumor suppressor, KLF11 [76]. Interestingly, a genome-wide association study (GWAS) from 7683 patients with pancreatic cancer and 14,397 controls found that one of the four identified SNPs to reach genome-wide significance was located near KLF14 [77].

Using the *Pdx1*-Cre/*Kras*$^{LSL\text{-}G12D}$ mouse model of PDAC precursor lesions in combination with cigarette smoke exposure, Edderkaoui and colleagues determined that inhibition of HDAC3 reverses the accelerated PanIN formation observed from smoking and thus is a major player in mediating the pro-cancer effects resulting from this exposure [78]. This effect is facilitated, at least in part, through HDAC3-mediated regulation of IL-6 production in cancer cells to influence macrophage function, specifically the pro-tumor type-2 macrophage (M2) phenotype, in the tumor microenvironment. Several HDAC inhibitors have FDA approval, including Vorinostat, Romidepsin, and Belinostat [79], and thus, most ongoing studies in the field are focused on their use as targeted epigenetic therapeutics in PDAC, which is the topic of a subsequent chapter dedicated to "Epigenetic Pharmacology." In summary, it is clear that HDACs play an important role in the maintenance of the proper balance of chromatin marks on a given promoter, and if this balance is altered, such as HDAC expression in pancreatic cancer, the expected global effect on promoters is daunting.

Histone H3-Methyl-K27 and Polycomb

Polycomb proteins silence gene expression by specific methylation of histone H3 on K27 [68, 80]. At the simple core of this pathway, polycomb group (PcG) proteins act via the stepwise recruitment of PRC2, containing the H3K27 methylase activity, to chromatin. Subsequently, the trimethyl-H3K27 mark deposited by PRC2 recruits the PRC1 complex, thereby completing the gene silencing complex formation. The enzymatic activity of the PCR2 complex involves the H3K27 histone methylase, EZH2, but requires a complex with Suz12 and EED to function. The PCR1 complex contains the oncogene BMI1, as well as HPC1–3, HPH1–3, SCMH1, and the methyl-H3K27-binding proteins, Cbx 2, 4, 6, 7, and 8. However, which of the Cbx proteins is active at different loci under different circumstances is not known.

The role of polycomb proteins in pancreatic cancer has elicited significant attention over the recent years. For instance, new polycomb proteins have been discovered in pancreatic cancer cells [81]. More importantly, studies have demonstrated that loss of trimethylation at H3K27, which is achieved by EZH2, is a predictor of poor outcome in pancreatic cancers [82]. In fact, together with tumor size and lymph node status, the level of trimethyl-H3K27 was found to have a strong and independent prognostic influence in pancreatic cancer. Nuclear accumulation of EZH2 was identified as a hallmark of poorly differentiated pancreatic adenocarcinoma, and this nuclear overexpression of EZH2 contributes to pancreatic cancer cell proliferation, suggesting EZH2 as a potential therapeutic target for the treatment of pancreatic cancer [83]. In samples obtained by endoscopic ultrasound-guided fine-needle aspiration (EUS-FNA), EZH2 expression was determined by immunohistochemistry to evaluate its use as a potential biomarker for treatment and disease prognosis [84]. However, EZH2 expression was heterogeneous and did not correlate inversely with E-cadherin expression as expected to serve as a hallmark of poorly differentiated pancreatic adenocarcinoma. Nevertheless, interest remains high for EZH2 as a therapeutic target in PDAC. Using the cerulein-induced model of pancreatic injury, EZH2 levels increase after injury, and this methyltransferase is required to promote the tissue repair process through inducing regenerative proliferation of progenitor cells [85]. With genetically engineered animal models, the same study revealed that EZH2 knockout impairs pancreatic regeneration and accelerates $KRas^{G12D}$-driven PanIN formation. Recent investigations found that activated CDK5 kinase is responsible for EZH2 phosphorylation, which is required for F-box and WD repeat domain-containing 7 (FBW7) to target EZH2 for ubiquitination and subsequent degradation [86]. As a result, this process suppresses EZH2 activity and thereby inhibits tumor migration and invasion of pancreatic cancer cells, not only highlighting the role of EZH2 overexpression present in PDAC samples, but providing additional therapeutic targets as well.

In terms of the PRC1 complex, a study on the ubiquitin E3 ligase Ring1B, a key component of PRC1 by catalyzing monoubiquitination of histone H2A at lysine 119 (H2AK119Ub1), and Snail, a transcriptional repressor and master regulator of epithelial-mesenchymal transition (EMT), demonstrated that elevated levels of these two molecules along with elevated monoubiquitination of H2AK119 are highly correlated with poor survival in PDAC [87]. On the other hand, reduction in CBX7 levels was associated with increasing malignancy grade in pancreatic adenocarcinoma and correlated with a loss of E-cadherin expression [88]. Conservation of CBX7 levels trended with longer patient survival rates, suggesting that loss of this polycomb protein contributes to a more aggressive pancreatic cancer phenotype. Moreover, CBX7 plays a role in suppression of cell proliferation, migration, and invasion, which is thought to occur in part through reducing PTEN/Akt signaling [89]. Pancreatic cancer stem cells, a small subset of distinct cancer cells with great proliferative potential and resistance to standard therapies, were identified to have upregulation of the PRC1 molecule Bmi-1, which enhances tumorigenicity and the function of the cancer stem cell population [90]. Interestingly, similar to CBX7, Bmi-1 influences the Akt signaling pathway, but by activating PI3K/AKT signaling

through the negative regulation of PTEN [91]. This mechanism was found to stimulate invasion and metastasis of the pancreatic cancer stem cells. Pancreas-specific inactivation of Bmi-1 in the *Pdx1*-Cre/*Kras*$^{LSL\text{-}G12D}$ murine model of pancreatic cancer initiation suggested that Bmi-1 is required for this process, in an Ink4a/Arf-independent manner [92]. Loss of Bmi-1 resulted in the upregulation of ROS, indicating that this PRC1 molecule regulates protection from excess ROS during neoplastic transformation, which is required for survival and progression. Thus, the association of this pathway with poor survival of patients affected by this disease renders this area of research one of paramount importance.

Mechanistically, one of the outcomes of aberrant polycomb regulation is the silencing of the *p16* gene, which could occur prior to DNA methylation, via altered direct recruitment of members of this family to the *p16* promoter sequence [93]. Upon studies in human cells, EZH2 and DNA methyltransferases (DNMTs) were found to physically and functionally interact, evidenced by the PRC2 subunits, EZH2 and EED, co-immunoprecipitating with all three human DNMTs and the co-dependency of certain target gene silencing requiring both EZH2 and DNMTs [94]. Therefore, the presence of polycomb proteins on the *p16* promoter can recruit DNA methylases which then further inactivate the expression of *p16* via DNA methylation (Fig. 1). However, whether histone H3K27 methylation and recruitment of DNMT to result in DNA methylation ultimately leads to permanent mutation/deletion of the gene or all mechanisms of *p16* inactivation are independent remains to be discovered.

Histone H3-Methyl-K9 and Heterochromatin Protein 1

As described in a prior section, HP1 binds methylated K9 of histone H3, causing transcriptional repression [68, 95]. This occurs through the N-terminal chromodomain of HP1, while the highly related C-terminal chromoshadow domain allows for dimerization of these HP1 molecules and serves as a docking site for various factors involved in a wide array of functions, from transcription to nuclear architecture. To mediate gene silencing via the formation of heterochromatin, HP1 isoforms must interact with different H3K9 histone methylases, G9a (EHMT-2), GLP (EHMT-1), and SUV39H1 [68, 95]. These methylases work in concert with HP1 in a circular manner to form silenced chromatin. When the methylases adds methyl groups to K9 of H3, this, in turn, forms an HP1 docking site on chromatin. Since HP1 also recruits the methylases, this cycle repeats, and the HP1–methylase pair can spread the formation of silenced chromatin to adjacent nucleosomes, causing long-term silencing of entire genes (Fig. 1).

Information regarding the function of HP1 proteins in both normal and tumor pancreatic cells is still emerging. However, HP1 proteins have altered expression in many different types of cancers, including breast, brain, ovarian, colon, and papillary thyroid cancers as well as leukemias [96]. Noteworthy, with the three human isoforms having over 80% similarity between them, the factors that influence these

differences remain unknown. Unfortunately, despite the identification of numerous HP1 binding partners, distinct signaling cascades that mediate the interaction with these proteins to ultimately "switch on" or "switch off" gene silencing remain largely unknown. Although the discovery of the previously mentioned HP1-mediated subcode [31] contributed to this understanding, it remains essential to carefully define these pathways to map useful networks of membrane-to-chromatin signaling cascades for better understanding of the regulation of activation, repression, as well as other cellular processes. The molecular mechanisms that operate as subcodes within the histone code trigger nuclear instructions imparted by H3K9 methylation, which are subsequently translated as silencing, and thus, potentially participating in the silencing of tumor suppressor genes.

One specific example of how the methyl-H3K9/HP1 type of chromatin dynamics can impact on the field of pancreatic cancer is the regulation of MUC1 expression. The sialylated form of MUC1 is overexpressed in invading and metastatic pancreatic cancer cells, but absent in normal pancreas, cases of chronic pancreatitis, and pancreatic ductal hyperplasia [97], lending this molecule to be an interesting target for immunotherapeutic strategies [98]. Strikingly, studies have recently demonstrated that a mechanism responsible for changes in the expression of MUC1, which can in turn make proposed vaccines less than optimal, is regulated by DNA methylation and H3K9 modification, which is bound by HP1, on the *MUC1* promoter [99]. Similar to polycomb, it is known that HP1 can recruit DNA methyltransferases [100], which can lead to the silencing of this important molecule for pancreatic cancer (Fig. 1). MUC1-negative cancer cell lines correlated with high DNA methylation and methyl-H3K9 levels, while MUC1-positive cell lines had low levels of these epigenetic marks. Increased expression of NFATc2 in advanced PanIN-2/PanIN-3 lesions and PDAC coincides with silencing of the $p15^{INK4b}$ tumor suppressor pathway, which mechanistically has been linked to recruitment of SUV39H1, to result in H3K9 trimethylation and subsequent binding of HP1γ [101]. Interestingly, the first genome-wide study on the epigenetic landscape, comparing matched primary and metastatic PDAC lesions collected by rapid autopsy, revealed widespread epigenetic reprogramming during the evolution of distant metastasis without the presence of metastasis-specific driver mutations [102]. This reprogramming presented as global changes specifically in histone H3K9 and DNA methylation within large heterochromatin domains, known as LOCKs, as well as regional changes in histone marks, such as acetyl-H3K27 at gene regulatory elements. Inhibition of the H3K9 pathway results in senescence of pancreatic cancer cells without inducing apoptosis, thereby reducing anchorage-dependent and anchorage-independent proliferation [103]. Furthermore, the combined inhibition of the Aurora kinase A oncogene with the H3K9 pathway impedes PDAC cell growth via a mechanism that, instead of senescence, involves perturbation of normal mitotic progression to end in mitotic catastrophe [104]. Therefore, chromatin dynamic-driven epigenetic changes have the potential to extend research beyond the minimal mutation paradigm to include other pathways that are also important for other key biological behaviors in pancreatic cancer.

Additional Nonhistone Chromatin Proteins as Epigenetic Targets

Other nonhistone chromatin proteins, such as the Sin3a scaffold, play a role in pancreatic cancer [105]. For instance, pancreatic cells express three different Sin3 proteins that are recruited by tumor suppressors, such as the Myc antagonist, Mad1, and KLF11, and these tumor suppressor proteins require binding to a Sin3a–HDAC complex to perform their function (Fig. 1). Thus, this system is both active and important for antagonizing pancreatic carcinogenesis. Furthermore, pathogenic mutations and structural variants have been discovered in several epigenetic regulator genes, resulting from whole genomic sequencing of 100 pancreatic cancer samples, including *KDM6A*, *ARID1A*, *ARID1B*, *PBRM1*, *SMARCA2*, *SMARCA4*, and *MLL2* [106]. Interestingly, *KDM6A*, which encodes for an H3K27me3 demethylase, was inactivated in as much as 18% of the pancreatic cancer patients. Another KDM6 family member, KDM6B, which also demethylates H3K27me3, has loss of heterozygosity in pancreatic cancer cells and its loss is associated with enhanced tumor sphere formation, as well as increased peritoneal dissemination and liver metastasis in vivo [107]. Thus, the future anticipates studies of these various complexes in the context of pancreatic cancer, which may reveal significant contributions to the initiation, maintenance, or spreading of this disease or to cancer-associated functions, such as stem cell maintenance, DNA repair, metastasis, and therapeutic response.

Noncoding RNAs and Pancreatic Cancer

Due to the discovery and increasing study of noncoding RNAs, including microRNAs (miRNAs), short interfering RNAs (siRNAs), piwi-interacting RNAs (piRNAs), and long noncoding RNAs (lncRNAs), a significant number of researchers are analyzing noncoding RNA signatures in pancreatic cancer. The best-characterized noncoding RNAs are miRNAs, which are endogenous noncoding RNA molecules approximately 21 nucleotides in length that have been found to play important roles in the regulation of genes in animals and plants via a process involving their pairing to the mRNAs of protein-coding genes to direct their post-transcriptional repression [108]. In fact, miRNAs are currently predicted to control the activity of approximately 30% of all protein-coding genes in mammals. Similar to coding transcripts, miRNAs are classified into oncogenic miRNAs and tumor suppressor miRNAs in relation to their function during tumorigenesis. In an early global profiling study, several miRNAs were identified as aberrantly expressed in pancreatic cancer or desmoplasia [109]. Interestingly, some of these have been previously reported as differentially expressed miRNAs in other human cancers, including miR-155, miR-21, miR-221, and miR-222, in addition to some novel ones not previously reported, such as miR-376a and miR-301. Typically, the most aberrantly expressed miRNAs were found to be downregulated in the tumor tissue. Several additional profiling studies have found miRNA deregulation in human PDAC. In another study, several miRNAs, including miR-205, −18a, −31, −93,

−221, and −224, were demonstrated to be overexpressed in primary neoplastic ductal cells and pancreatic cancer cell lines, representing promising biomarkers for pancreatic cancer [110]. Furthermore, 26 miRNAs were identified as the most significantly misregulated in pancreatic cancer and the analysis of only two miRNAs, miR-217 and -196a, allowed discrimination between normal and neoplastic tissues, further supporting the potential use of miRNAs for the diagnosis of pancreatic cancer. Bloomston and colleagues also performed a global analysis to compare miRNA profiles of normal pancreas, chronic pancreatitis, and pancreatic adenocarcinoma [111]. In 90% of the tested samples, 21 overexpressed and 4 downregulated miRNAs were capable of differentiating pancreatic cancer from benign pancreatic tissues via cross validation. Additionally, 15 miRNAs demonstrated increased expression and 8 showed decreased expression, which could distinguish pancreatic cancer from chronic pancreatitis with 93% accuracy. Noteworthy, a subgroup of 6 miRNAs was able to discriminate node-positive disease between long-term survivors and patients who would succumb to the disease within 24 months. Poor survival of pancreatic cancer, with a median survival of 14.3 months versus 26.5 months, could be predicted with 95% confidence through high expression of miR-196a-2.

Certainly, the studies of miRNAs in pancreatic cancer in general have grown significantly over the last decade. However, with increased interest and focus on identifying circulating biomarkers in PDAC as a noninvasive, cost-effective, and reliable means to detect and/or monitor the disease, it is important to discuss the use of miRNAs in this context, as well as the contribution of circulating miRNAs to the disease. miRNAs can be detected in human plasma, circulating as free RNAs, either bound to hAgo2 or included in exosomes, which are stable and protected from endogenous RNase activity [112]. The first relatively large study performed by multiple independent centers reported that 29 circulating miRNAs from pretreatment blood samples collected before clinical or surgical intervention had the potential to differentiate PC cases from healthy volunteers [113]. Of these, 13 miRNAs were selected for further validation. While their diagnostic value was not significantly different than CA19–9, this report represented a proof-of-principle that circulating miRNAs can serve as potential biomarkers for early pancreatic cancer. A meta-analysis performed on 29 published studies, including a total of 2225 patients and 1618 controls, to evaluate the diagnostic accuracy of circulating miRNAs for pancreatic cancer diagnosis found multiple miRNAs to have a relatively high diagnostic value compared to single miRNA diagnosis [114]. A retrospective screen of early stage pancreatic cancer patients and controls detected 15 differential candidate miRNAs in plasma samples from pancreatic cancer patients at diagnosis [115]. However, these circulating miRNAs, alone or in combination, were not significantly altered in prediagnostic plasma samples from an early detection case-control cohort, suggesting that these miRNAs emerge late in disease development and would not function for early detection. Studies of this nature are still in their relative infancy, and if reliable circulating miRNAs are identified for early detection and/or monitoring disease progression, this noninvasive and cost-effective window into an epigenetic signature has a promising future in clinical application.

In addition to miRNAs, another class of noncoding RNAs that have elicited attention as novel drivers of tumorigenesis are long noncoding RNAs (lncRNAs). lncRNAs are longer than 200 nucleotides in length with their genomic location mainly in intronic and intergenic regions [116]. These RNAs are transcribed by RNA polymerase II, even with similar mRNA structures, such as a 5′ cap and a 3′ poly (A) tail, and based on the proximity to protein-coding genes are classified as antisense, sense, bidirectional, intronic, and intergenic lncRNAs. lncRNAs are believed to function in a variety of ways, including as cis- or trans-regulators of gene activity, as scaffold elements, guides, or decoys for chromatin-modifying complexes, or as gene enhancers. In respect to pancreatic cancer, recent studies have revealed several lncRNAs with differential expression in pancreatic cancer, including well-known lncRNAs such as H19, HOTAIR, HOTTIP, and MALAT-1, among others [117]. Even though most non-protein-coding transcripts belong to this class of RNAs, representing more than 20% of the genome, their highly diverse structures and functions provide a source of much understanding that remains unknown regarding these molecules in both, health, and disease.

In summary, the revised paradigm for the better understanding and promoting further research in pancreatic cancer, besides taking into consideration only mutations and deletions, as well as promoter DNA methylation, now includes both chromatin dynamics, noncoding RNAs, and nuclear shape (Fig. 5a, b). It is noteworthy to underscore that although more work on chromatin dynamics is needed to understand pancreatic cancer development and phenotype, little has been done about the role of nuclear shape in this disease. Therefore, the purpose of this model is to further fuel a new era of experiments that expand the scope of the field from a DNA-centric paradigm to a holistic and more inclusive model, which takes into consideration protein-mediated epigenetics, noncoding RNA-mediated effects, and the biology of the nucleus as an altered organelle in the progression of pancreatic tumors (Fig. 5a, b).

Conclusion

Increasing studies on chromatin dynamics are unveiling the existence of robust machineries that can mediate epigenetic changes in pancreatic cells. The research community needs to focus not only on somatic genetics, since this mechanism certainly does not represent the full story of alterations in gene expression for pancreatic cancer. This important fact has led to the design of a more comprehensive model that widely includes the emerging data in the field of chromatin dynamics and nuclear shape. Guided by this model, the knowledge gathered on this disease can be more accurately mapped to a progression paradigm that will not doubt impact on many areas of pancreatic cancer research and practice. The era of epigenetics has emerged strongly with well-justified and energetic beginnings, which will continue into a frontier area for pancreatic cancer research. The reversibility of the epigenetic changes, in itself, makes the journey worthwhile; however,

further insights into the mechanisms behind pancreatic cancer make the journey indisputable.

Box 1 Key Research Points

- The field of epigenetics has evolved from the fusion of studies on RNA polymerase II transcription and chromatin. The current theoretical framework in this field has been distilled from different paradigms, which have evolved during almost half a century with some replacement of each other.
- Pancreatic cells are excellent models for developing knowledge of three types of transcriptional events, namely basal transcription, activated transcription (e.g., growth factor-inducible), and tissue-specific gene expression (e.g., secretory granule enzymes).
- Studies on chromatin dynamics, including noncoding RNAs as well as nuclear structure and shape in pancreatic cells continue growing. The emerging data from these studies are benefiting not only this field, but extending the knowledge of the biology of other cells in the body. In addition, current evidence links these phenomena to development, homeostasis control and diseases. Therefore, this area may constitute one of the most promising in basic and translational pancreatic cell research.

Box 2 Future Scientific Directions

- Epigenetic mechanisms that are involved in stem cell biology, organ morphogenesis, and pancreatic cancer development constitute a new and very promising frontier. In particular, the discovery of how signaling and chromatin together determine cell fate during development and regeneration as well as how epigenetics contributes to the cancer phenotype is of paramount importance, biologically and pathobiologically.
- Cell-specific mechanisms for regulating gene expression are well advanced only in acinar cells. Therefore, more studies are necessary to understand the biology of ductal cells. In addition, epigenetic mechanisms are known to take part in the processes of pattern formation, such as branching morphogenesis, which is better understood in *Drosophila melanogaster* where chromatin-mediated effects play a significant role in this process. Therefore, studies on chromatin may aid in better understanding the formation of the pancreatic duct and its branching, which is of significant biomedical interest.
- Animal models for studying the genetic mechanisms necessary for the progression of pancreatic cancer have been a major contribution to the field of pancreatic cancer. Models for studying epigenetic effects in pancreatic cells must follow to understand the role of epigenetics in the pancreas at the whole organism level.

Box 3 Clinical Implications

- The revised "holo-genetic model for pancreatic cancer" covered in this chapter may help to guide future research in pancreatic cancer in a similarly productive manner to the guidance provided by the original genetic model for pancreatic cancer.
- It would be important to map key epigenetic changes that occur in the sequence of PanIN lesions along with the known mutations, to develop a better understanding of their potential mechanistic interrelationship. Therefore, development of new markers with good predictive value for whether an earlier PanIN has the potential to transform into another more malignant lesion would be beneficial.
- The most relevant characteristic of epigenetics, which is extremely attractive for therapeutic purposes, is its reversibility. Due to the difficulties surrounding gene replacement, it is likely that gene therapy for pancreatic cancer will remain, at least for a while, a hard-to-reach ideal. Therefore, due to its reversibility, epigenetics may provide attainable useful tools for chemoprevention and chemotherapy.
- In general, nuclear proteins and noncoding RNAs, which are shed by tumors into the bloodstream and are specific to detect pancreatic cancer, may be another prolific area of investigation with a great impact on diagnostics.

Cross-References

- ▶ Animal modeling of Pancreatitis-to-Cancer Progression
- ▶ Epigenetic Pharmacology
- ▶ Hedgehog Signaling Plays a Dual Role in Pancreatic Carcinogenesis
- ▶ Mouse Models of Pancreatic Exocrine Cancer
- ▶ Precision Medicine Based on Next-Generation Sequencing and Master Controllers
- ▶ Role of Tumor-Stromal Interactions in Pancreatic Cancer Invasion and Metastases
- ▶ The Molecular Pathology of Precursor Lesions of Pancreatic Cancer

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