Replicating DNA at top speed : chromatin at risk

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Replicating DNA at Top Speed

Chromatin at Risk

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PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Universiteit Maastricht,
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Prof. mr. G.P.M.F. Mols
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Contents

Chapter 1	7
General Introduction	
Aim of the thesis	11
Chapter 2	13
Replicating DNA at Top Speed - Chromatin at Risk. (New tales from aging bones).	
Chapter 3	29
A novel in vivo model to study enchondral bone formation; HIF-1 α activation and BMP expression	
Chapter 4	45
The immediate early gene EGR1 controls epigenetic reprogramming through Polycomb Group proteins during chondrogenesis.	
Chapter 5	79
Polycomb Repressive Complex 1 Coordinates Enhanced Replication and Transcription in Transit Amplifying Chondrogenic Progenitors.	
Chapter 6	113
Proliferative Capacity Defines Sensitivity to Valproic Acid During Chondrogenesis.	
Chapter 7	131
General Discussion	
Summary	139
Samenvatting	143
Dankwoord	147
Curriculum Vitae	151
List of publications	153
Abbreviations	155
Colour figures	159

Chapter 1

General introduction

Introduction

DNA stores all genetic information that is required for a cell to manufacture the molecules it needs to function. Although most somatic cells in a human body contain identical DNA sequences, the vast variety of cellular phenotypes implies that the information stored in the DNA sequence on its own is not sufficient to explain cell diversity and function. Cells depend on the acquisition and the maintenance of epigenetic traits to acquire and maintain a specific phenotype. An epigenetic trait is formally defined as “*a stably heritable phenotype resulting from changes in a chromosome without alterations in the DNA sequence*”¹. Genomic DNA is a major constituent of chromatin, a complex structure consisting of DNA, and all associated proteins and RNA. The nucleosome is the fundamental unit of chromatin, consisting of two copies of the 4 core histones (H2A, H2B, H3 and H4) with 147 base pairs of DNA wrapped around it. N-terminal histone ‘tails’ protrude from chromatin and are subject to a wide variety of posttranslational modifications. Over recent years, many enzymes and chromatin associated proteins have been identified that can write, erase and/or read these modifications, providing an epigenetic register of, for instance, transcriptional activity. Increasing evidence suggests that epigenetic processes dynamically regulate partitioning of the genome in ‘active’ and ‘inactive’ chromatin. Chromatin can undergo dynamic changes in structure, resulting in various states of compaction, which either facilitate or block accessibility of the DNA template. Chromatin structure modification is an important regulatory aspect of all DNA templated processes, including e.g. transcription, replication and repair².

Epigenetics

Increasing insights into the molecular mechanisms underlying epigenetic regulation reveals an incredibly complex apparatus that has major implications for development and disease. Although epigenetic regulation controls several processes, regulation of transcription is understood best, and this occurs at the level of RNA, DNA and proteins.

At the DNA level, cytosines in CpG dinucleotides were found to be methylated by DNA methyltransferases (DNMTs) and promoter DNA methylation by and large correlates with transcriptional silencing³.

At the RNA level, non-coding RNAs were identified as transcriptional repressors⁴. Perhaps the best known non-coding RNA is *Xist*: *Xist* covers the outside of the inactive X-chromosome and thereby maintains the transcriptionally inactive state⁵. Another mechanism by which non-coding RNAs silence gene expression is RNA interference (RNAi): microRNA’s and small interfering RNA’s (siRNAs) bind RNA through (sometimes imperfect) sequence homology thereby regulate gene expression through blocking gene transcription, regulating RNA translation, RNA processing or RNA stability⁴. For example, non-coding RNA can bind messenger RNA (mRNA) to prevent translation or to activate the RNAi pathway to degrade mRNAs by recruiting the Risc complex to mRNAs. Interestingly, binding of non-coding RNAs to specific mRNAs was also shown to enhance translation.

At the protein level, N-terminal histone tails in nucleosomes are subject to numerous posttranslational modifications (*i.a.* (mono-, di-, or tri-) methylation, phosphorylation, acetylation, ubiquitylation) by a diversity of enzymes, such as histone methyltransferases (HMTs), kinases, histone acyltransferases (HATs) and ubiquitylases ². Other enzymes are specialized in removing histone modifications, e.g. histone demethylases (HDMs), phosphatases, histone deacetylases (HDACs) and deubiquitylases ². Proteins can recognize and bind to specific modifications through specialized domains, forming chromatin-associated multi-protein complexes with relevant epigenetic activity. In addition to the various types of modifications that can occur, histones can be modified at multiple sites and may be recognized in combination. In the context of transcription, these collective histone modifications constitute an epigenetic index of gene activity. How epigenetics precisely regulates gene activity is incompletely understood. Certain modifications strongly correlate to transcriptional activity, or inactivity, although no perfect correlation has been found to date. Together, this array of writers, erasers and binders of epigenetic modifications suggests complex and dynamic epigenetic regulation of gene activity.

Polycomb Group proteins

Polycomb Group (PcG) proteins are best known as transcriptional repressors. First identified in *Drosophila melanogaster* as important regulators of segmentation, PcG complexes maintain expression boundaries of homeobox (*Hox*) genes within the Homeotic gene clusters (*HOM-C*) along the antero-posterior (AP) axis. *Hox* genes are important regulators of segment identity in the fly. It is clear that PcG proteins exert a repressive function and that PcG proteins and their targets are conserved throughout evolution. Also in mammals PcG control segmentation: *Hox* genes determine vertebral identity along the AP-axis. PcG-mutations in the mouse cause vertebral identity changes and other skeletal defects ⁶. PcG proteins form large multiprotein complexes that combine intrinsic with associated epigenetic activities. PcG repressive complex 2 (PRC2) for instance harbors Eed, SUZ12 and the HMT EZH2, which harbors a SET domain with HMT activity for lysine 27 on histone 3 (Figure 1). This PcG associated posttranslational modification is recognized and bound by specific chromobox proteins (CBX) in the PRC1 complex that also contains PHC, BMI1, RING1 and RNF2 (Figure 1). In addition the PRC1 complex harbors E3 ligase activity for lysine 119 of H2A. Both PRCs are associated with many other factors relevant for regulation of transcription. In the fruit fly larvae PRC2 is believed to initiate PcG-based gene silencing, whereas PRC1 takes over as a maintenance complex. Although PcG are known as transcriptional repressors, how PRCs control repression of promoter activity is mechanistically far from understood.

Figure 1 Schematic overview PRC1 and PRC2 complexes

Enchondral ossification

Enchondral ossification is a multistep process in which the ossified skeleton is formed from a cartilaginous matrix. The local microenvironment plays an important role in regulation of the different steps of Enchondral ossification. Locally produced soluble factors, such as insulin-like growth factors, bone morphogenetic proteins, fibroblast growth factors and many others ⁷ regulate the different stages of chondrocyte and osteocytes maturation.

Cartilage is a dense connective tissue, consisting of collagen, proteoglycan and elastin fibres, excreted by chondrocytes. Cartilage is characterized by the absence of blood vessels, and thus the hypoxic environment. Therefore chondrocytes receive nutrients and oxygen by diffusion from synovial fluids, and depend heavily on hypoxia-inducible factor 1 (HIF-1) and anaerobic metabolism to function and survive ⁷.

Longitudinal growth of bones is accomplished by two growth plates (epiphysary regions) that move away from each other as condensations of mesenchymal (stem/progenitor) cells differentiate into chondrocytes. The growth plates are arranged in distinct regions that reflect the different stages of chondrocyte differentiation. The first phase of chondrocyte differentiation is characterized by activation of the proliferative capacity of resting chondrocytes and longitudinal growth occurs. Next, the proliferating chondrocytes become flattened and packed in condensed clusters. Through a phase of pre-hypertrophy, the chondrocytes terminally differentiate into hypertrophic chondrocytes. In addition to excretion of the cartilage matrix, hypertrophic chondrocytes secrete *i.a.* VEGF to induce the formation of blood vessels, and TGF β to attract mesenchymal cells and to differentiate these cells into osteocytes. As blood vessels and osteocytes enter the cartilage matrix, chondrocytes undergo programmed cell death and osteocytes calcify the extracellular matrix to form bone.

Aim and outline of this thesis

Mechanisms underlying PcG mediated transcriptional repression are beginning to unravel. In addition, although poorly understood, PcG proteins have been associated with replication and DNA repair. However, many questions about Polycomb group biology remain: how are PcG proteins regulated themselves? How are PcG proteins involved in mediating gene-environment interactions? How are PcG proteins targeted to cell-type specific targets? What is the mechanism behind PcG mediated transcriptional repression? What is the role of PcG in replication and DNA repair? What is the role of PcG in enchondral ossification?

We here specifically focus on the role of PcG proteins in chondrogenesis. PcG mutant mice display skeletal malformations along the AP-axis, associated with deregulation of *Hox* gene expression boundaries. However, very little is known about the direct involvement of PcG proteins in enchondral ossification. The working hypothesis of the research described in this thesis is that PcG proteins mediate gene-environment interactions, thereby allowing the epigenomic landscape to change and to facilitate chondrogenic differentiation. The research described in this research aimed to elucidate the role of PcG proteins and underlying molecular mechanisms in chondrogenic differentiation.

In chapter 2, a detailed review of our current understanding of epigenetic regulation of transcription and replication, with a particular focus on PcG proteins, is presented. Chapter 3 describes a novel *in vivo* model to study enchondral ossification in relation to gene-environment interactions, with important relevance to alternative tissue repair strategies in regenerative medicine. In chapter 4, *Egr1* was identified as an important immediate early transcription factor, rapidly induced upon chondrogenic differentiation. We find that *Egr1* directly controls epigenetic remodelling required for chondrogenesis, and present evidence for interaction with PcG function at several levels. Chapter 5 describes the role for *Bmi1* in regulating transcription and replication during transit amplification of differentiating chondrocytes. In chapter 6, we show that the epigenetic drug valproic acid induces replication stress during transit amplifying chondrocytes, leaving the cells incapable of fulfilling the differentiation program. Finally, in chapter 7 the results obtained in these studies are discussed.

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Chapter 2

Replicating DNA at Top Speed - Chromatin at Risk. (New tales from aging bones).

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Abstract

Polycomb Group proteins have been associated with numerous cellular processes that use chromatin as a template, including transcription, replication and DNA repair. The link between Polycomb function and DNA replication is a long-standing one, yet poorly understood. Recent observations position Polycomb proteins at the cross road of transcription and replication and begin to offer explanations for cellular and morphological phenotypes. Here we review this emerging role for Polycomb in the context of development and cancer.

Introduction

Lineage commitment and terminal differentiation in the context of development are dependent on acquisition and maintenance of heritable epigenetic traits. An epigenetic trait is formally defined as “a stably heritable phenotype resulting from changes in a chromosome without alterations in the DNA sequence”². Although gradually unfolding, the epigenetic mechanisms underlying stable inheritance of cell fate decisions (*i.e.* in mitosis and meiosis) are still far from being completely understood. Chromatin remodeling underlies all DNA-templated cellular processes such as replication, transcription and repair. Continuous redefinition of chromatin states by covalent modification of DNA (*i.e.* CpG methylation) and/or chromatin associated proteins (*e.g.* post-translational modifications of core histones), allows a cell to undergo cell fate determination and to function in its micro-environment, *i.e.* to respond to constantly changing physiological or metabolic conditions. Lineage commitment and early differentiation are likely to inflict massive strain on the genome, which, under these conditions, is simultaneously transcriptionally redefined and replicated. Both the transcriptome and the genome are subject to dynamic changes throughout differentiation. Within the context of differentiation, however, the underlying epigenetic mechanisms that help tuning replication to transcription and *vice versa* remain poorly understood. It is conceivable that differential epigenomic states functionally partition the genome and help guide cells through differentiation, establish and maintain novel physiological states, while allowing genome duplication without compromising genetic integrity and genome stability. Thus DNA transcription, DNA replication and DNA repair require coordination if they are to occur from one and the same template. Perhaps, predictably, transcription, replication and repair ‘machineries’ share basal components. It is likely that such proteins and the regulatory pathways they control evolved to tune these processes in the context of differentiation in multi-cellular organisms. Polycomb Group proteins are connected to many processes that require chromatin structure remodeling, among which transcription, replication and repair^{3,4}. Although biochemical processes relevant to epigenetic regulation of transcriptional repression by PcG complexes are becoming increasingly clear, how exactly cellular memory is installed and whether and how this connects to DNA replication remain enigmatic. We here review how specialized pathways and chromatin structure control help cells cope with several important chromatin-based processes (like

transcription and replication) that, although seemingly in conflict with each other, often occur simultaneously in the context of differentiation and development. Specific focus will be on the potential role of Polycomb Group (PcG) proteins in these control mechanism.

Epigenetics in transcription

Perhaps the most studied and mechanistically relatively best understood example of epigenetic control of a DNA-templated process is that of transcription. Transcription, production of RNA from DNA, is a process highly coordinated by a variety of proteins surrounding the key factor in transcription: RNA polymerase. Polyadenylated RNA synthesis requires formation of a preinitiation complex (PIC) by RNA polymerase II (RPOL II) with the general transcription factors (GTF) TFIID, TFIIA and TFIIB ⁵. GTF direct RPOLII to these core elements and transcription is initiated by ATP-dependent TFIIF helicase activity, which uncoils and denatures the DNA allowing RPOLII to bind single stranded DNA. To date, in eukaryotes seven core promoter elements have been identified (e.g. TATA box), which comprise specific DNA sequences required for promoter function and for proper assembly of the PIC ⁶. Both initiation of transcription and elongation of RNA by RPOLII are phosphorylation dependent events ^{7,8}. Access to DNA by local chromatin structure remodelling events is essential for initiation and elongation. This is controlled at the epigenetic level and includes both covalent histone modifications and nucleosome remodeling machineries ⁹. Although the order of these events is currently under debate, they essentially recruit transcriptional activators and expose DNA binding elements.

Select post-translational modifications (PTM) of histone proteins are linked to specific biological processes. Examples include histone variant H2AX phosphorylation in the context of DNA damage responses and specific histone H3 acetylation and methylation events that are often associated with activation and repression of transcription, respectively ^{3,7,10}. Histone modifications often occur and function in conjunction, for example, histone acetylation and methylation events are collectively responsible for nucleosome displacement and transcriptional activation ¹¹. Overall, the epigenome is defined by a combination of molecular mechanisms at all three macromolecular levels, DNA, RNA and protein, which are thought to collectively constitute a highly complex register of gene status ⁴. Some modifications occur in correlation with higher-order chromatin structures. For example, as opposed to euchromatin, condensed constitutive heterochromatin is often associated with H3K9me3. Compared to euchromatin, heterochromatin often displays distinct subnuclear localisation, is replicated later during S-phase and heterochromatic DNA repair requires additional components ¹². Although a putative inductive role for H3K9me3 in chromatin condensation is neither proven nor clear, the correlation does illustrate an important concept: the eukaryotic genome is functionally partitioned by the epigenome.

Polycomb in Transcription

Polycomb Group (PcG) proteins are best known as transcriptional repressors. First described in *Drosophila melanogaster* as important regulators of segmentation, it is clear that PcG genes, and hence protein structure, their repressive function and their targets are conserved throughout evolution. PcG proteins form large macromolecular complexes that combine intrinsic with associated epigenetic activities. PcG repressive complex 2 (PRC2) for instance harbors EZH, a SET domain protein with histone H3K27-directed methyltransferase (HMT) activity. This PcG associated PTM is recognized and engaged by specific chromobox proteins (CBX) in the PRC1 complex. Both PRCs comprise many other factors relevant for regulation of transcription (see table 1). In the fruit fly larvae PRC2 is believed to initiate PcG-based gene silencing, whereas PRC1 takes over as a maintenance complex¹³. Although experimental models, seemingly with very few exceptions, define PcG as transcriptional repressors, how PRCs control repression of promoter activity is mechanistically far from completely understood. The now somewhat antiquated notion that PcG silencing is based on chromatin compaction (i.e. higher order structures) and consequently reduced accessibility for transcription factors is based on steric principle, finds limited experimental support¹⁴. Recent studies identifying PcG association with the general transcription factory machinery provide new insights into PcG silencing mechanisms. In the fruit fly, PcG act by assembling on Polycomb Responsive Elements (PREs), DNA sequences that can regulate silencing of a nearby promoter in a PcG-dependent fashion¹⁵. PcG silencing only slightly reduces accessibility of restriction enzymes to the promoter and binding of TATA-binding-protein (TBP) and Rpol2 to the *HSP26* promoter was not lost, arguing against chromatin compaction as a major feature in PcG-mediated repression. *Drosophila* PRC1 proteins interact with several TBP-associated factors (TAFs), that complex with the general transcription factor TFIID^{16,17}. Furthermore, the *Xenopus eed* homologue, a PRC2 member, interacts with TAF_{II}32, a key component of the TFIID complex in *Xenopus*¹⁸. Relevantly, Rpol2 still associates with PcG-silenced promoters but is inhibited in its ability to initiate transcription¹⁶. Trithorax group proteins are epigenetic activators known to contain histone methyl transferase activity for H3K4, which is correlated with active transcription. Interestingly, trithorax proteins activate transcription by counteracting PcG function and by promoting the early phase of transcription elongation¹⁹. Although this points to PcG silencing taking place at primary chromatin fibers, the seemingly paradoxical co-occurrence of PcG proteins and RPOLII at repressed promoters, and conversely, the finding that numerous H3K27me3-associated promoter are transcriptionally active^{20,21} indicate there is more to PRC-mediated repression than meets the eye. An overview of PcG-associated proteins involved in regulation of transcription is presented in table 1.

Epigenetics in replication

DNA replication serves to duplicate DNA in cells in preparation of mitosis. DNA replication is controlled by mechanisms highly conserved throughout evolution. The first step in replication initiation is licensing of replication by recruitment of multiprotein complexes to discrete foci within the nucleus, called replication origins. Replication origins acquire replication competence during G1 phase in eukaryotes. Binding of the origin recognition complex (ORC) functionally defines DNA replication origins by formation of a pre-replication complex (pre-RC) through complexation with Cdc6 and Cdt1, a replication licensing factor that recruits the MCM2-7 helicase complex. Subsequently, additional proteins including Cdc45 and DNA polymerases join the pre-RC to form the replication fork. The assembly of these multiprotein complexes at replication origins signals the beginning of S-phase. The helicase activity of the complex unzips the DNA, and next the ssDNA is bound by the heterotrimeric protein RPA preventing the DNA from winding back on itself. The ring-structured replication factor PCNA is loaded onto DNA and associates with topoisomerases and replicative DNA polymerases. By sliding along the DNA ahead of the replication fork PCNA enhances the processivity of enzymes in the replication fork ^{22,23}.

Termination of replication involves Geminin (Gmnn). Gmnn binds free Cdt1, and prevents access of the MCM complex to Cdt1, thereby preventing the initiation of novel pre-RC's. As such Gmnn prevents inappropriate reinitiation of replication, and thus ensures that DNA is replicated only once. Gmnn expression commences at the onset of S-phase and its expression is maintained until the end of mitosis; subsequent inactivation of Gmnn then allows the cell to prepare for a new round of replication ^{23,24}.

Several lines of evidence implicate chromatin structure in spatial and temporal regulation of DNA replication. As for transcription, chromatin needs to be made accessible for DNA replication. (Hetero)chromatin needs to be decompacted and DNA needs to unwind for replication to occur. Although very little is known about chromatin states around ORCs and pre-RC complexes, this is likely to involve some form of epigenetic regulation. Cell cycle dependent chromatin remodelling is most dramatically visible at the G2/M transition, when chromosomes condense. Although relatively little is known about epigenetic changes that accompany this condensation, some well-known histone-PTMs precede chromosome condensation, e.g. H3S10, H3S28 and H2AX phosphorylation. Many chromatin associated proteins are also phosphorylated in mid/late S and released from chromatin. Combined phosphorylation may be required for ensuring chromosome condensation and/or new transcriptional states are epigenetically defined at this time point in the cell cycle when chromatin is assembled. Not only DNA is replicated faithfully, epigenetic information is also transferred from one cell to the other. Chromatin-proteins, like histones, are believed to play a crucial role in this cross-generational transcriptional memory. DNA replication commences at the onset of S-phase and is subject to dynamic programming. Classic nucleotide analogue pulse-labelling experiments have established that DNA replication is differentially regulated throughout the nucleus, defining characteristic S-phase replication patterns in space and

time. In 1963 Hay and Revel showed that euchromatin is replicated early, whereas heterochromatin is replicated late in S-phase²⁵. In line with this, DNA replication occurs early in S-phase within genomic regions which are transcriptional active, whereas late replicating DNA is found in transcriptionally inactive regions²⁶. Goldman showed that housekeeping genes, active in all cells are replicated during the first half of S-phase²⁷. Also, tissue-specific genes are replicated early in tissues in which their transcriptional activity is high, whereas non-tissue specific genes are replicated late²⁷. As this differential replication is maintained over subsequent rounds of replication and cell division²⁸, it seemed reasonable to assume that structural organization of chromatin defines both transcriptional status and replication timing. Hence replication timing profiling may provide information about the developmental status and lineage potential of a cell. Indeed, recent comparisons of replication timing of lineage specific and non-specific genes between pluripotent embryonal stem cells (ESCs) and uni-potent T lymphocytes or neural cells revealed that replication timing is closely linked to lineage restriction and developmental status^{20,29}.

The most direct evidence for coordinate tuning of transcription and replication comes from 3D-fluorescence imaging studies in mouse and human diploid fibroblasts. Euchromatic DNA replication and transcription are constrained to defined nuclear areas, indicating that an underlying genomic architecture, likely involving epigenomic marking, segregates the two processes in time and place³⁰. Combined the data shows that replication timing is not a static process but that it dynamically shifts across the genome in the contexts of commitment and terminal differentiation^{20,29}. Thus, replication timing is an intrinsic cellular feature and is closely linked to transcription. A perhaps logical consequence of this is the notion that transcription and replication need to be tuned to each other during replication. This raises a number of questions: 1) is replication timing a consequence of chromatin structure regulation in the context of transcription? or 2) *vice versa* does replication timing provide a cellular mechanism to transmit epigenetic information to the next generation, with transcriptional regulation as a consequence? 3) What epigenetic mechanisms are responsible for definition and proper coordinated regulation of both transcription and replication throughout S-phase?

The first cause-or-consequence issues have been studied extensively in the mammalian *β-globin* locus. The *β-globin* locus is replicated from a single bidirectional origin, firing early in S-phase in erythroid cells, and late in all other cell types³¹. Transcriptional activity of the *β-globin* locus is controlled by a *locus control region* (LCR). Although loss of the LCR at the *β-globin* locus significantly reduces transcription of the *β-globin* genes chromatin structure, measured by DnaseI sensitivity, and replication timing are unaffected³². This suggests that at least for the *β-globin* locus, replication timing is not defined by transcriptional status. However, whether specific histone modifications at the LCR are involved in transcriptional regulation of the *β-globin* locus, and whether those participate in replication timing is currently unknown.

To begin to probe causal and consequential relationships between transcription and replication timing, it was proposed that chromatin structure is (re)established by virtue of differential

expression of chromatin modifiers (epigenators; ²) at specific time points during S-phase ^{26,33}. Proteins involved in assembly of euchromatin would be expressed early in S-phase where proteins involved in heterochromatin formation would be expressed late in S-phase. In support of this, the *de novo* DNA methyltransferase DNMT1, a transcriptional repressor implicated in heterochromatin formation, targets DMAP1, a transcriptional co-repressor, to replication foci during the entire S-phase. Also HDAC2 associates with DNMT1 and DMAP1, although this interaction was restricted to late S-phase ³⁴. Thus, factors involved in the formation of transcriptionally repressed epigenetic state are recruited to chromatin during the period in S-phase when heterochromatin is duplicated. It is conceivable that e.g. micro-environmental cues (*i.e.* morphogens, growth/differentiation factors, cell-cell contacts) may provide the signalling required for (re)activation of epigenators and tissue specific transcription factors to lineage relevant genes and networks ². At the bases of coordinated regulation of DNA-templated processes, the assumption that euchromatin is readily available and accessible for replication, and is consequently replicated early, whereas the heterochromatin fraction is less accessible and needs to be remodeled before replication can take place may be a workable starting point. X-chromosome inactivation provides a model to study epigenetic mechanisms in replication timing and transcriptional inactivation. Differentiating female embryonic stem cells inactivate one of two X chromosomes through mechanisms involving a non-coding RNA, called *Xist*. *Xist* covers the entire inactive X (Xi) chromosome ³⁵. The Xi is hypoacetylated on histone 3 and 4, shows increased H3K9 and DNA methylation, and relevantly, Xi is replicated late in S phase ³⁶. Interestingly, mutations in the DNA methyltransferase *dnmt3b* in female lymphoblasts are associated with DNA hypomethylation and shift Xi-linked gene replication to earlier time points in S-phase ³⁷. In keeping with this, inhibition of DNA methylation results in both reactivation of gene expression of Xi genes and advanced replication timing ³⁸. Although epigenetic mechanisms are clearly involved, not one chromatin modification perfectly correlates with replication timing, hence there must be some degree of flexibility to replication timing and/or additional mechanisms must be responsible for regulation of replication timing in relation to transcription.

Polycomb in replication

During development expression patterns have to be maintained through several rounds of cell divisions. Histone modifiers and modifications provide a mechanism by which cellular memory of gene expression can be inherited to next cell generations. Polycomb proteins and their counterparts Trithorax proteins are known to be involved in transmitting epigenetic information to next generations during replication ³⁹. During every cell cycle, when DNA is replicated in S-phase, chromatin-associated proteins and histone modifications are diluted, and need to be re-established on the newly formed daughter strands. Current models imply that modified parental histones are randomly distributed across the two replicating daughter strands ⁴⁰. Interestingly, besides for PRC1, H3K27me3 acts as a recognition mark for the PRC2 complex; this suggests a possible

mechanism for transmission of H3K27me3 marks by PRC2 to newly assembled nucleosomes ⁴¹. Recent studies also suggest a more direct role for PcG proteins in DNA replication. *Drosophila* mutants for PRC1 members *Ph*, *Pc* and *Psc*, and the Enhancer of Trithorax and Polycomb (ETP) gene *Asx* show severe chromatin segregation defects in syncytial embryos ⁴². Although poorly understood, these and other earlier observations provided an association between PRC-function and DNA replication ⁴². Importantly, *Drosophila* Grp/Chk1 and Chk2 (DmChk2) mutants also accumulate segregation defects, as a result of failure to activate checkpoint (metaphase to anaphase delay) in response to DNA damage or incompletely replicated DNA ⁴³. These raises the question whether the accumulation of segregation defects in PcG mutant *Drosophila* is the direct consequence of problems in DNA replication and whether a DNA damage response is involved (please see discussion below).

Direct and genetic interactions exist between PcG proteins and proteins involved in regulating DNA replication. Topoisomerase II (Topo2), well-known for its role in replication, and Barren (BARR), a potential activator of Topo2 ⁴⁴, both associate with genomic PcG target sequences in *Drosophila* ⁴⁵. In line with the finding that Topo2 and Barren bind PREs, they directly bind to the PcG protein Polyhomeotic (Ph). In addition, Cramped (CRM), a *Drosophila* PcG protein, localizes to the nucleus during S-phase and CRM expression disappears at mitosis. The cell cycle-dependent localization resembles that of PCNA and suggested a functional relationship. Indeed, CRM and PCNA colocalize on salivary gland polytene chromosomes and a double mutant for *crm* and *pcna* (*Drosophila mus209* allele), displays an exaggerated phenotype in anteroposterior and distal-proximal transformations. Besides shared function, more importantly, this illustrates that defective replication is a determining factor in abnormal segmentation ⁴⁶. In further support of functional kinship, a transient association of Gmnn with the mammalian PRC1 members Scmh1 and Phc1/Mphc/Rae28 was recently discovered ⁴⁷. And finally, the mammalian PcG protein Bmi1 was identified as a direct interactor of the replication licensing factor CDC6: the transition of late to early replication of the INK4A/ARF locus in senescing embryonic fibroblasts underscores the functional association between PcG and replication timing ⁴⁸. Overall, whilst experimental proof in this area will undoubtedly expand, the collective data suggests that PcG function is directly implicated in regulation of replication. An overview of PcG-associated proteins involved in regulation of replication and repair is presented in table 1.

Coordinated regulation replication and transcription by PcG

Although PcG proteins are classically associated with transcriptional repression, mounting evidence supports a broader role for PcG in other DNA templated processes besides transcriptional regulation. In *Drosophila*, regulatory elements of *lab-4* and *mcp* are enriched for both Polycomb (PC) and Trithorax (TRX) early in embryogenesis, while seemingly in conflict with the classic view that PRC alter chromatin for transcriptional repression, both genes are transcriptionally active ⁴⁹. Analogously, bivalently marked promoters in murine embryonic stem

cells, carrying both repressing (H3K27me3) and activating (H3K4me3) marks, were found to be transcriptionally active, albeit at low levels ²¹. We recently found evidence for significant transcriptional induction of putative PcG-target genes *in vitro* and *in vivo*, despite persistence of H3K27me3 marks; *in vitro* phospho-dependent release of PRC1-complex members correlated with activation of expression (⁵⁰+ *in preparation*). These findings begin to show that the role of H3 trimethylation, and possibly PRC1 function, exceeds that of transcriptional silencing only.

Cells undergo replication stress under conditions that affect replication fork progression during S-phase. As stalled replication can be mutagenic and induce cell death, several pathways have evolved to repair damaged DNA and to get collapsed replication forks back on track ^{51,52}. Activation of sophisticated intraS-phase checkpoints allows cells to resolve replication stress and DNA damage, and to complete S phase to allow cells to proceed to mitosis ⁵³. The majority of DNA damage in replicating cells, even under normal conditions, probably comes from highly frequent collision between replication forks and transcription machineries, as both processes move along the same template ^{51,54}. Hence, these processes need to be tightly regulated to prevent DNA damage. Under conditions of rapid proliferation, e.g. in response to enhanced mitogenic signalling by, among others, oncogenic E2Fs, cMyc, Ras, Braf, and RUNX, the increased replication rate puts a tremendous strain on chromatin. Upon initial oncogene-induced hyperproliferation, sustained expression of oncogenes in fibroblasts activates a senescence response termed oncogene-induced senescence (OIS) ^{55,56}. In support of the notion that under hyperproliferative conditions, cells are unable to cope with the stress that the increased demand for DNA replication puts on chromatin, cells expressing oncogenic RasV12, for example, amass replication-dependent double strand DNA breaks (DSB) and accumulate stalled replication forks; senescent cells harbour senescence-associated DNA-damage foci (SDF), containing ATM, NBS1, ATR, ATRIP and RPA ⁵⁶. Activation of a DNA damage response (DDR) is instrumental in the induction of OIS, as knock-down of various DDR proteins (ATM, ATR, Chk1, Chk2 and p53) rescues proliferation ⁵⁶. Intriguingly, co-expression of Bmi1 in RasV12 expressing cells bypasses senescence to a large extent ⁵⁷. Although mechanistically not worked out in detail, analogy with oncogenic cooperation between c-Myc and Bmi1 points to a possible involvement of INK4A/ARF suppression by Bmi1 ⁵⁸. Recent data from our group support a role for PRC1 in replicative control and begins to shed some light on underlying mechanisms. In analogy to expression of oncogenes in fibroblasts, activation of differentiation *in vitro* often sets off a transient hyperproliferative phase commonly referred to as transit amplification TA: transit amplifying cells (TAC) provide the necessary expansion of progenitor populations to provide for adequate tissue repair/replacement from limited numbers of mobilized stem cells (SC). Although hard to pinpoint *in vivo*, TA has been suggested to underlie the rapid regeneration of tissues, among which intestinal villi from SCs located in the crypts of Lieberkühn ⁵⁹. One of the few places in the body where TAC are readily identifiable is the proliferative zone in the developing skeleton: during endochondral ossification (the formation of bony skeletal elements from cartilage scaffolds) histologically distinctive tracts of rapidly

proliferating cells are readily visible⁶⁰. TA can be mimicked *in vitro*, in chondrogenic models: the mesenchymal stem cell ATDC5 will undergo a proliferative burst upon induction of differentiation⁶¹. Despite the characteristic effect of many PRC1 null-mutations on antero-posterior skeletal morphogenesis in mice⁶², a direct role for PRC1 in chondrogenesis was never established. Unexpectedly, PRC1-deficient ATDC5 cells fail to undergo TA, and activate an intraS-phase block in response to massive accumulation of DNA damage. PRC1-KD cells are very sensitive to replication poisons; accumulate DSB at sites of *de novo* DNA synthesis and ultimately senesce, exposing a causal relationship between defective PRC1-function and replication induced DNA damage (this thesis).

An obvious question is: how does PRC1 prevent replication stress? A number of observations points in the direction of coordinated regulation of transcription and replication during TA. Whereas G1 nuclei typically stain brightly for Ser5-phosphorylated Rpol2 (pRpol2), cells progressing through S-phase normally undergo a transition from genome-wide transcriptional activity to complete shut-down of transcription initiation at mid/late S-phase. Co-staining for BrdU and pRpol2 revealed enhanced BrdU incorporation at sites where transcription initiation was low, or put differently, low transcriptional activity at site of DNA replication (³⁰, this thesis). Remarkably, this functional genome partitioning is completely lost in S-phase arrested Bmi1-deficient cells. In addition, cells carrying DSB display a generally deregulated transcription, which is also reflected in comparative expression array analysis in the context of differentiation: transcription levels of many genes were consistently upset throughout differentiation; in addition, genes irrelevant to chondrogenesis were induced, whereas other genes that should have been induced were not. Although deregulation of specific pathways awaits further analysis, it is clear that redefinition of transcription and replication activity are closely coupled during chondrogenesis and that somehow PRC1 is required to facilitate differentiation under hyperproliferative conditions.

Recently, PRC1 was identified as an E3 ligase for Gmnn: Rnf2/Bmi1-mediated ubiquitylation of Gmnn targets it for proteasomal degradation. *In vivo*, loss of the PRC1 protein Phc1, through which Gmnn is associated to PRC1 and a direct interactor of Bmi1 and Rnf2, results in stabilization of Gmnn and blocks haematopoietic stem cell proliferation⁶³. Stabilization of Gmnn indeed prevents cell from completing their cell cycle⁶⁴. To explain the leukopenia in Phc1-null mice, PRC1-mediated control of Gmnn expression was suggested to affect the proliferative potential and/or differentiation capacity of HSC⁶⁵. The earlier described physical interaction between PcG complexes with elements of transcription factories as well as replication machineries, in addition to the physical and biochemical interactions between PRC1 and Gmnn, suggest a mechanism for PRC1-mediated orchestration of transcription in respect to replication and/or *vice versa*. We here propose a model in which PRC1/Gmnn acts as a molecular switch between transcriptional and replicative choices in mid-S phase (figure 1): H3K27me3-mediated recruitment of the core complex CBX/Rnf2/Bmi1 installs repressive H2AK119ub1 marks, which interfere with transcription initiation, possibly through recruitment of additional PRC (associated) proteins¹. Local presence of

Rnf2/Bmi1 E3 ligase activity would target Gmnn for degradation, relieving a potential replication block. Conversely, loss of Bmi1/Rnf2 stabilizes GMNN, hence blocking replication, yet permitting transcription. Chromatin dissociation of Bmi1/Rnf2 is likely controlled by post-translational modification (PTM). In line with this idea, we recently observed a positive correlation between PRC-phosphorylation, PRC1/chromatin dissociation and Gmnn stabilization under various conditions of cell stress (JWV, YT unpublished observations). Stress signaling induces chromatin dissociation of PRC1 members (Bmi1, Ring and CBX proteins; ⁵⁰). In contrast, Phc1/Rae28 ⁵⁰ undergoes a nuclear redistribution under these conditions and may provide a docking point for Gmnn stabilization.

As indicated before, Gmnn levels gradually increase toward late S-phase and chromatin-bound Gmnn blocks rereplication of DNA at the in G2/M. Increased Gmnn-chromatin association correlates with PRC1 dissociation from chromatin during mid S-phase ⁶⁶. Relevantly, Bmi1-deficient chondrogenic progenitors arrest in midS-phase and display significantly increased Gmnn chromatin association (this thesis). The inverse correlation between Gmnn and Bmi1/Rnf2 chromatin association during mid-S is in perfect agreement with the ubiquitin-mediated induction of Gmnn proteolysis by Bmi1/Rnf2 ⁶⁵. Relevantly, the most dramatic replication-retiming happens at mid-S-phase in the context of differentiation, when replication origins are spatially redefined and replication timing and transcription are re-tuned ²⁶. The dynamic relocation of PRC1 and Gmnn during this phase suggests a functional connection with replication timing in the context lineage commitment and differentiation from a pluri- or multipotent stem cell.

Of note, the primary task of H3K27me3 (and possibly H3K4me3) in the proposed model would not be to mediate transcriptional repression (or activation, respectively) *per se*. Rather global (and local) histone trimethyl (and possibly additional) marking may provide the required epigenomic context for tuning transcription and replication. Our observation that cell stress consistently co-induces global H3 trimethylation at K4, K27 and K9, supports the idea that these marks regulate more than transcription. Hence, PcG and trxG-associated epigenetic marks may functionally partition the genome and help coordinate DNA-templated processes, for example in the context of development.

Concluding remarks

Early observations in *Drosophila melanogaster*, although incompletely understood, provided an early indication of a link between PRC-LOF phenotypes and DNA replication ⁴². We here provide a tentative explanation for the replication defects in PRC-mutants: PRC1 orchestrates chromatin-based processes, such as increased transcriptional reprofiling and enhanced DNA replication during transit amplification in the context of differentiation. The consequence of this observation in relation to the etiology of PRC1 phenotypes *in vivo* is as of yet unclear. PRC1 proteins classically maintain *HOX* gene expression boundaries set by segmentation and pair-rule genes, and skeletal phenotypes correlate with defective maintenance of *HOX* gene expression boundaries ⁶⁷. In line

with published data on regulation of proliferation by *HOX*-genes, in addition to long-standing speculations about contribution of a proliferative component to axial and appendicular phenotypes in *HOX* mutants, it is possible that proliferation and *HOX* gene expression both contribute to abnormal replication⁶⁸. However, since no two single PRC1 mutants affect the same *HOX* genes; although by and large, most PRC1-null mutants display relatively similar posterior transformations, this automatically begs the question whether *HOX* deregulation may be consequential rather than causal in this context. Multiple additional interesting questions arise: What is the exact molecular nature of functional genome partitioning? Does replication timing determine transcriptional activity, or *vice versa*? Is coordination of transcription and replication subject to clustered regulation, i.e. groups of genes or networks in the context of lineage commitment, and how exactly are PRCs implicated in these matters? What post-translational modifications and protein interactions help direct PRC activity toward transcriptional repression or replication? Can the findings reported herein be readily projected onto embryonal stem cells, *i.e.* does failure to properly coordinate simultaneous DNA templated processes contribute to self-renewal defects? And, in the context of OIS, is PRC1 inactivation involved in OIS or, conversely could Polycomb overexpression in cancer be required for protection during hyperreplication? Chromatin has been called the most relevant physiological substrate in a cells' life. Coordinated regulation of processes like transcription, replication and DNA repair is important to prevent DNA damage and help maintain genome integrity, cell identity and provide regenerative capacity. As all these processes use one and the same template, DNA, it should come to no surprise that many regulatory components are shared, including Polycomb group proteins and geminin. An important future challenge will be to determine the exact molecular mechanisms and contributions of Polycomb Group proteins at the cross road of chromatin templated processes, like transcription and replication.

<i>Drosophila</i>	Mammals	Associated proteins	Process
E(z)	Ezh1/Ezh2	Xnp ⁶⁹	DNA repair
Esc	Eed	TAF _{II} D (<i>Xenopus</i>) ¹⁸	Transcription
Su(z)12	Suz12		
Pc	Cbx4, Cbx6, Cbx7, Cbx8	TBP ¹⁷	Transcription
Pcl	Phf1	Rad50, Smc1, Dhx9 and p53 ⁷⁰	DNA repair
Ph	Phc1/Phc2/Phc3	Tbp ¹⁷ , Gmnn ⁴⁷ Topo2, Barr ⁴⁴	Transcription/Replication
Sce/dRing	Ring1A/Rnf2	Tbp ¹⁷	Transcription
Psc	Bmi1/ Mel18	Tbp ¹⁷ , Gmnn ⁴⁷ , Cdc6 ⁴⁸	Transcription/ Replication
Pho	YY1	Tbp ¹⁶	Transcription
Smc	Smch1	Gmnn ⁴⁷	Replication
Crm		Pcna ⁴⁶	Replication
	Rybp	Mdm2 ⁷¹	DNA repair

Table 1 Overview PcG binding proteins**Figure 1** **Figure 1.** Proposed model for segregation of transcription and replication by PcG proteins.

H3K27me3-mediated recruitment of the core complex CBX/Rnf2/Bmi1 installs repressive H2AK119ub1 marks, which interfere with transcription initiation, possibly through recruitment of additional PRC (associated) proteins¹. Local presence of Rnf2/Bmi1 E3 ligase activity would target Gmnn for degradation, relieving a potential replication block. Conversely, loss of Bmi1/Rnf2 stabilizes GMNN, hence blocking replication, yet permitting transcription.

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Chapter 3

A novel in vivo model to study enchondral bone formation; HIF-1 α activation and BMP expression

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Abstract

Numerous growth and transcription factors have been implicated in endochondral bone formation of the growth plate. Many of these factors are up-regulated during hypoxia and downstream of Hypoxia-Inducible Factor (HIF)-1 α activation. However, the specific function of these factors, in the context of oxygenation and metabolic adaptation during adult periosteal endochondral bone formation, is largely unknown. Here, we studied HIF-1 α and the possible roles of (HIF-1 α related) growth and transcription factors in a recently developed in vivo model for adult periosteal endochondral bone formation.

At different phases of periosteal endochondral bone formation, mRNA levels of Transforming Growth Factor (TGF)- β 1, Bone Morphogenetic Proteins (BMP)-2, -4, and -7, Indian Hedgehog (Ihh), Parathyroid Hormone-related Protein (PTHrP), Sox9, Runx2, HIF-1 α , Vascular Endothelial Growth Factor (VEGF), periostin (POSTN), and Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) were evaluated with RT-real time-PCR. Also protein levels of TGF- β 1, BMP-2, -4, and -7, HIF-1 α , and POSTN were examined. During the chondrogenic phase, the expression of Sox9, Ihh, and HIF-1 α was significantly up-regulated. TGF- β 1 mRNA levels were rather constant, and the mRNA levels of BMPs were significantly lower. Immunohistochemical detection of corresponding gene products, however, revealed the presence of the proteins of TGF- β 1, BMP-2, -4, and -7, HIF-1 α , and POSTN within the chondrocytes during chondrogenesis. This discrepancy in gene expression between mRNA and protein level for TGF- β 1 and the different BMPs is indicative of post-transcriptional regulation of protein synthesis. HIF-1 α activation and up-regulation of GAPDH support a hypoxia-induced metabolic shift during periosteal chondrogenesis. Our model recapitulates essential steps in osteochondrogenesis and provides a new experimental system to study and ultimately control tissue regeneration in the adult organism.

Introduction

Endochondral bone formation is a multistage process; early commitment of mesenchymal stem cells (MSC) to a chondrogenic cell fate is accompanied by morphological changes and activation of a cartilage specific gene program. At progressive differentiation stages toward osteogenesis, chondrocytes resume proliferation, mature into hypertrophic chondrocytes, and then calcify their surrounding matrix and undergo programmed cell death¹. Simultaneously, angiogenesis is stimulated and the calcified cartilage is replaced by bone. Transforming Growth Factor (TGF)- β 1 and members of its superfamily, among which are the Bone Morphogenetic Proteins (BMPs), play an important role in endochondral bone formation and fracture healing². BMPs are known to interact with the Indian Hedgehog (Ihh)/Parathyroid Hormone related Protein (PTHrP) pathway and exert their function, at least in part, via expressional regulation of Sox9, an essential transcription factor in chondrogenesis¹⁻⁷. In addition, hypoxia, as a micro-environmental determinant, is thought to promote differentiation of mesenchymal cells along a chondrocyte pathway, in part by activating Sox-9 via an HIF-1 α -dependent mechanism⁸. Although conditions are hypoxic within the endochondral growth plate⁹, not much information is available on oxygenation and metabolic adaptation during periosteal callus formation. Several observations directly link oxygen tension to osteochondrogenic differentiation. The transcription factor Runx2 (Aml3, Osf2/Cbfa1) is required for differentiation of osteoblast progenitors during the final stages of endochondral bone formation and is also downstream of BMP signalling^{10,11}. Runx2 mRNA is subject to IRES-dependent translational control, which is thought to permit continued translation under conditions suboptimal for cap-dependent translation (e.g. under low oxygen concentration)¹². The cell adhesion protein periostin (POSTN, Osf2—not to be confused with Osf2/Cbfa1) is highly homologous with β igH3/ β igH3 and is induced by TGF- β 1 and hypoxia¹³⁻¹⁶. POSTN may play a role in the recruitment and attachment of osteoblast precursors and is specifically found in periosteum and the periodontal ligament¹⁴. The mRNA expression for this protein is highly up-regulated during fracture healing and also increases with mechanical stress in periodontal ligament tissues¹⁷⁻¹⁹. Most of these findings stem from in vitro models. A possible connection between oxygenation and expression of these growth and transcription factors during periosteal endochondral bone formation in vivo has so far not been studied. Studies on endochondral bone formation in vivo often use whole embryos or growth plates as paradigms^{5,6,20}. In addition, fracture healing models in adult rodents are used in which the bone marrow is penetrated^{21,22}. To date, experimental models for endochondral bone formation in adult organisms have, to our knowledge, not been described, whereas such models are likely relevant in respect to clinical situations. We have recently established a novel in vivo model for osteochondrogenesis, in which periosteum is damaged without breaking the bone and accessing the bone marrow²³. Periosteum contains pluripotent progenitor cells that harbor MSC characteristics^{24,25} and can differentiate to chondrocytes and osteoblasts during normal bone growth and fracture healing^{26,27}. We demonstrated the chondro-regenerative capacity of periosteal MSC²³. This novel model can be used to study the function of periosteum during cartilage and

endochondral bone formation. In addition, the model is expected to provide important mechanistic insight for tissue engineering of cartilage and/or bone and for fracture healing²³. The aim of this study was to evaluate expression of essential factors, among which is the hypoxia-responsive factor HIF-1 α , during the in vivo generation of adult periosteum derived cartilage and bone.

Materials and methods

Surgical procedures

Six-month-old female New Zealand White rabbits were used for this study. European and Dutch laws on animal experimentation were strictly followed throughout the study, and the animal experimental protocol was approved by the Maastricht University committee for animal experiments. Surgical procedures were performed under general anesthesia and with the use of sterile techniques. Anesthetic induction was obtained with 35 mg/kg ketamine hydrochloride and 5 mg/kg xylazine hydrochloride administered by intramuscular injection. Anesthesia was maintained by means of a mixture of 2% halothane and oxygen/nitrous oxide delivered by an automatic ventilator using an especially designed mask. All rabbits received a preoperative intramuscular injection with antibiotics (10 mg/kg ceftiofur sodium, Pharmacia and Upjohn, Woerden, The Netherlands). Postoperative analgesia provided by administering 0.05 mg/kg buprenorphine intramuscular once a day for 2 days. Bilaterally, the skin and the overlying fascia were opened over the upper medial tibia. A piece of approximately 7×15 mm of the periosteum was dissected. The harvested tissue of five rabbits was used to assess expression levels of the genes of interest in undifferentiated periosteum (t=0 day). The overlying fascia was closed with Polysorb 2.0 sutures (Tyco Healthcare, USA). Special care was taken that the tendon of the semitendinosus muscle was sutured on top of the lower, short side of the rectangular periosteum defect. Hereafter the skin was closed with Polysorb 4.0 (Tyco Healthcare, USA). At days 10, 20, and 40 postoperative, the reactive tissue of five rabbits per time point was harvested. For this purpose, the rabbits were anesthetized as described above. The reactive tissue was dissected with a scalpel or osteotome (40 days). Again the overlying fascia and skin were closed with Polysorb (Tyco Healthcare, USA). Specimens were cut in two halves, one part was used for RNA isolation, and the other was fixed in 4% paraformaldehyde, dehydrated and embedded in paraffin.

RNA isolation

Immediately after harvest, the tissue was frozen in liquid nitrogen and stored at -80°C. The frozen tissue was cooled further in liquid nitrogen and pulverized using a microdismembrator S (Bauer, Goettingen, Germany). The resulting powder was collected in TriZol reagent, and RNA was extracted according to the protocol of the manufacturer (Invitrogen, Breda, The Netherlands). Quantity and purity of the RNA were determined 260/280 nm absorbance measurements. The RNA samples were subjected to RNase-free DNase digestion to remove any genomic DNA and to

agarose gel electrophoresis to assess the intactness of RNA. Complete removal of genomic DNA was verified by PCR.

Rabbit primer design

Since sequence information on the rabbit genome is limited, conserved human and murine gene sequences were determined to design primer sets for *Ihh*, *Runx2*, *Sox9*, Collagen Type II and Type X and *POSTN* by using Blast2 software (www.ncbi.nlm.nih.gov). PCR primer sets were designed for homologous gene sequences and tested on rabbit cDNA samples (Supplemental table 1). The identities of the PCR products were confirmed by direct sequencing and comparison with known human and mouse genes and then submitted to GenBank (AY633663, AY598932, AY598933, AY598934, AY598935, AY598936, AY598937).

Semi-quantitative reverse transcription-polymerase chain reaction

Although multiple real time primer sets were tested, the expression of Collagen Type (COL) X, TGF- β 1 and *Ihh* could only be determined with a semiquantitative RT-PCR. To this end, we performed PCR reactions on serially diluted cDNAs using primer sets for *Ihh*, TGF- β 1, COLX, either alone or in combination with a primer set for 28S. 28S cDNA amplification was also performed separately. Ethidiumbromide signals were quantified using a GELDoc 1000 system with Multi-Analyst imaging analysis (Bio-Rad, CA, USA). Marker mRNA levels are expressed relative to 28S rRNA. Two hundred nanograms of heat denatured RNA was reverse transcribed with 100 U MMLV reverse transcriptase (Promega, WI, USA) in 50 mM Tris-HCl (pH 8.3), 75 mM KCl, and 3 mM MgCl₂ and 10 U RNasin (Promega, WI, USA). Two microliters c-DNA from each sample was amplified in 10 μ L PCR reaction mixture. For semi-quantitative expression measurements, 200 ng cDNA was diluted in five-fold increments (i.e. undiluted, 5 $\times$, 25 $\times$, 125 $\times$, 625 $\times$ diluted). Eight microliters of each PCR sample was run on a 2% agarose gel containing 0.5 mg/mL ethidiumbromide (Invitrogen, Breda, The Netherlands). Positive bands were visualized and quantified with a Geldoc 1000 system using Multi-Analyst software (Bio-Rad, CA, USA). Values were normalized to 28S rRNA expression.

Real time PCR assays

Real time PCR assays were performed in triplicates with qPCR mastermix for SYBR® Green I (Eurogentec, Seraing, Belgium) in 96-well optical plates with primer concentrations of 300 nM. Oligonucleotide sequences for Collagen Type I and II, aggrecan, BMP-2, -4, and -7, periostin, PTHrP, VEGF, 28S rRNA, GAPDH, *Sox9*, and HIF-1 α (Supplemental table 2) were based on the gene sequences which were submitted to or obtained from GenBank (Supplemental table 1) using Primer Express 2.0 software (Applied Biosystems, Foster City, CA, USA). cDNA was used for the real time quantitative PCR reactions using an ABI PRISM® 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA, user bulletin #2). Serially diluted standard curves were

included to quantify the samples. PCR conditions were: 2 min at 50°C, 10 min at 95°C, 40 cycles for 15 s at 95°C, and 1 min at 60°C followed by a melt curve 15 s at 95°C ramp to 60°C for 1 min, followed by 20 s at 95°C. Data were analyzed using Sequence Detection Software version 1.7 (Applied Biosystems, Foster City, CA, USA). Values were normalized to 28S rRNA.

Staining protocols

Paraffin sections were deparaffinated, hydrated and stained with thionine for 10 min. After washing and drying, coverslips were mounted using Entellan (Merck-Europe, Darmstad, Germany). For immunostaining, (paraffin-embedded) sections were deparaffinated and hydrated. Primary antibodies were diluted in PBS as follows: goat-polyclonal anti-BMP-2 (Santa Cruz, CA, USA, sc-6895) 1:25; goat-polyclonal anti-BMP-4 (Santa Cruz, CA, USA, sc-6896) 1:12.5; goat-polyclonal anti BMP-7 (Santa Cruz, CA, USA, sc-9305) 1:800; goat-polyclonal anti-TGF- β 1 (R&D Systems Inc, MN, USA) 1:30; mouse-monoclonal anti-periostin (5H8) 1:600 [28,29]; mouse monoclonal anti-HIF-1 α (Abcam Limited, Cambridge, UK, ab463) 1:50; and mouse monoclonal anti-Collagen Type II (II-II6B3, diluted 1:50; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA). Prior to the incubation with the goat-polyclonal anti-TGF- β 1, sections were placed in 250 mL Antigen Retrieval buffer (Dako Cytomation BV, Glostrup, Denmark) supplemented with 4.5 mL 0.1M citric acid+0.5 mL 0.1M Na citrate and incubated for 5 min in a microwave at 750 W. After incubation with the primary antibody, sections were washed with PBS and incubated with an appropriate biotin-labeled secondary antibody. Subsequently, sections were washed with PBS and incubated with the avidin–biotin binding complex with horseradish peroxidase (HRP) or alkaline phosphatase (ALP) (Dako Cytomation BV, Glostrup, Denmark). Diaminobenzidine (DAB) (Merck-Europe, Darmstad, Germany) was used as a substrate for HRP and Vectastain (Vector Laboratories, Burlingame, USA) for ALP. The sections were counterstained with hematoxylin dried and coverslipped.

Statistical analysis

Differences in mRNA levels were analyzed using the Mann–Whitney U test with a Hochberg post hoc correction test. Statistical significance was assigned for $p < 0.05$.

Results

Dissection of periosteum induced reactive tissue (periosteal callus—PC) which demonstrated cartilaginous characteristics at 10 and 20 days postoperative (PO) and osteogenic at 40 days (PO) as previously published²³. To validate the new osteo/ chondrogenic model, we mapped gene expression profiles during PC formation and differentiation and compared these to published data.

Fig. 1. Gene expression profiles throughout periosteal callus formation and differentiation (n=5 per time point). mRNA expression level of Collagen Type II (COL2;A), aggrecan/AGC (B), Sox9 (F), COLX and Ihh (G), and HIF-1 α (I) are significantly up-regulated during periosteal chondrogenesis (t=10). No significant mRNA up- or down-regulation is observed for COLI (B), TGF- β 1 (C), Runx2 (F), and periostin/POSTN (H) during follow-up. BMP-2, -4, and -7 are significantly downregulated during chondrogenesis (D, E). VEGF expression is up-regulated 20 days post-surgery (J). Quantitative RT-PCR expression measurements are normalized to 28S ribosomal RNA. Relative COLX, Ihh, and TGF- β 1 mRNA levels were measured by semi-quantitative RT-PCR (see Materials and methods for details). Briefly, relevant marker gene cDNAs were either separately amplified or in combination with 28S rRNA. SD is indicated by error bars.

Matrix and adhesion molecules in PC

As expected, in undamaged periosteum ($t=0$), Collagen Type II (COL2) expression was not detected. At 10 days PO, a significant up regulation of this marker for chondrogenesis was clearly detectable (Fig. 1A). Forty days post-surgery, COL2 expression had almost returned to basal level (Fig. 1A). A comparable expression profile was seen for aggrecan (AGC). Collagen Type I (COL1) was not significantly up- or downregulated (Fig. 1B). Thionine staining and immunohistochemical detection of COL2 correlated nicely with mRNA expression levels: no AGC and COL2 expression was detectable in control periosteum ($t=0$; not shown). Ten and twenty days PO, thionine staining indicated the presence of Glycosaminoglycans (GAG, i.a. AGC) and immunohistochemical detectable COL2 confirmed the expression of these proteins (Fig. 2A). Twenty days PO, ossification of the PC was first observed at the site attached to the tibial cortex (Fig. 2B). Forty days PO, full ossification was observed and only remnants of cartilaginous matrix molecules were detected (Fig. 2C). mRNA levels of the adhesion molecule periostin (POSTN) were not significantly up- or down-regulated during follow-up (Fig. 1C). In good agreement with this, a positive immunohistochemical staining for POSTN was observed in periosteum. This adhesion molecule was found intra- and peri-cellular in both the cambium layer and the fibrous layer of periosteum ($t=0$) (Fig. 3A). The matrix of the overlying fibrous layer remained positive during follow-up. At 10 days PO, POSTN was predominantly found in both hypertrophic and immature chondrocytes. The intensity of staining and number of POSTN positive cells were higher in chondrocytes located at then outside of the newly formed cartilage (Fig. 3C). Twenty days PO, only a few of the chondrocytes stained positive for POSTN. At 40 days PO, the adhesion molecule could only be detected in some of the bone marrow cells (Fig. 3D).

Growth factors in PC

Throughout PC formation and differentiation, relative expression of TGF- β 1 did not significantly change (Fig. 1D). BMP-2, -4, and -7 mRNA were down-regulated (2.4; 8.9; 3.7 decrease respectively) during the chondrogenic phase (Figs. 1E,F). Immunohistochemical staining of TGF- β 1 confirmed protein presence in periosteum. TGF- β 1 was found predominantly peri-cellular in both the cambium layer and the fibrous layer of periosteum (not shown). BMP-2, -4, and -7 proteins were not detected in normal periosteum. Ten days PO, BMP-2, -4, and -7 were clearly detectable in the chondrocytes in the newly formed cartilaginous tissue; intense staining for all BMPs was specifically seen in the pre-hypertrophic chondrocytes. Fig. 4D only shows BMP-2 staining, a similar staining was observed for BMP-2, -4, and -7; however, BMP-4 positive cells were detected less frequent and appeared to be more restricted to the hypertrophic zone (Figs. 4A, B). Ten days PO, TGF- β 1 was also found in both hypertrophic and mature chondrocytes and the overlying fibrous layer (not shown). Twenty days PO, BMP-2 and -4 could no longer be detected by

immunohistochemical staining (Fig. 4C). The intensity of BMP-7 staining was lower; a positive staining was only found in some hypertrophic chondrocytes and some osteoblasts in the newly formed trabecular bone (not shown). At this follow-up, the staining intensity for TGF- β 1 also decreased and was only found in the newly formed trabecular bone. Forty days post-surgery, the newly formed bone marrow was positive for TGF- β 1 and BMP-2, -4, and -7 (Fig. 4E, only BMP-2 is shown as example). Newly formed trabecular bone and bone marrow stained intensely for BMP-7; only a few cells were positive for BMP-2.

Fig. 2. Immunohistochemical detection of Collagen Type II in periosteal reactive tissue (callus); (A) 10 days post-surgery, this collagen is present in the newly formed cartilage (a thionine stained section is enlarged). (B) At 20 days post-surgery, the presence of Collagen Type II decreases at the zone of calcification (ZC and bone formation. (C) 40 days post-surgery, remnants of Collagen Type II are still visible (brown). Sections counterstained with hematoxylin. Original magnification (A) $\times 16$; (B) $\times 16$; (C) $\times 16$; insert in (A) $\times 100$.

Fig. 3. Immunohistochemical detection of periostin/POSTN in periosteum. (A) POSTN is detected (brown color) especially in the cambium layer (CL) but is also found in the fibrous layer (FL) of periosteum. (C) 10 days post-surgery, the number of POSTN-positive cells is higher in the peripheral chondrocytes (PCh) compared to the chondrocytes localized centrally (CCh). (D) 40 days post-surgery, POSTN is present in the newly formed bone marrow (BM). (B) Immunohistochemical staining of HIF-1 α shows its presence (red) in chondrocytes, 10 days post-surgery, no HIF-1 α staining is found in hypertrophic chondrocytes (arrows). Original magnification (A) $\times 200$; (B) $\times 200$; (C) $\times 200$; (D) $\times 200$.

Fig. 4. Immunohistochemical detection of BMP-4 in periosteal callus; (A) 10 days post surgery, chondrocytes stain positive (red) for BMP-4. (B) is an enlargement of the box in (A). (C) 20 days post-surgery, BMP-4 is neither detected in chondrocytes nor in the zone of calcification (ZC). Sections counterstained with hematoxylin. Immunohistochemical detection of BMP-2 in periosteal callus; (D) 10 days post-surgery, chondrocytes stain positive (red) for BMP-2. (E) 40 days post-surgery, BMP-2 is detected in the newly formed bone marrow (arrows), the overlying fibrous layer is indicated with FL. Original magnification (A) $\times 200$; (B) $\times 400$; (C) $\times 200$; (D) $\times 400$; (E) $\times 100$.

Key lineage specific signaling and programming molecules in PC.

The expression of the transcription factor Sox9 was significantly up-regulated (14 increase) at 10 days PO compared to $t=0$ (Fig. 1G). At 20 and 40 days PO, the expression level of Sox9 gradually decreased. No significant up- or down-regulation was observed for Runx2 (Fig. 1G). At 40 days post-surgery, then mRNA expression level of this transcription factor returned to the same level as detected in periosteum ($t=0$; Fig. 1G). In contrast to Parathyroid Hormone-related Protein (PTHrP) (Fig. 1G), Indian Hedgehog (Ihh) was not expressed in periosteum (Fig. 1H). During the chondrogenic phase ($t=10$) a significant up-regulation was seen for Ihh. At all later stages during osteogenesis, mRNA levels decreased for both these genes (Figs. 1G, H).

Oxygenation and angiogenic marker genes

A simultaneous increase in the mRNA expression level of GAPDH (2.6-fold increase) and a significant increase in expression level of HIF-1 α (3.4-fold increase) were detected at 10 days PO (Fig. 1I). Immunohistochemical detection of HIF-1 α protein was reflected in the mRNA expression profile; HIF-1 α was only detectable in the chondrocytes present at 10 days post-surgery. Mature chondrocytes and early hypertrophic chondrocytes stained positive for HIF-1 α (Fig. 3B). The protein was not detected in late hypertrophic chondrocytes (Fig. 3B). At 20 days PO, a few chondrocytes in the outer layer stained positive for HIF-1 α . In periosteum and at 40 days PO, HIF-1 α protein could not be detected by immunohistochemical staining. VEGF mRNA levels expression increased 20 days post-surgery (2.7-fold increase) (Fig. 1J).

Discussion

We here report on a first analysis of lineage specific marker gene expression in a novel in vivo model for osteo/chondrogenesis in full-grown animals. The model makes use of experimentally induced local damage to the periosteum. The damaged periosteum responds by callus formation; within this periosteal callus (PC), MSC-like activity is mobilized, which subsequently supports and recapitulates several well-described stages of chondrogenesis and osteogenesis. During the chondrogenic phase of PC formation, we find that cells of the cambium layer first commit to a chondrogenic cell fate; mRNA of the transcription factors Sox9, Ihh, is up-regulated and cartilage matrix molecules like aggrecan and Collagen Types II and X are produced. This stage is followed by chondrocyte hypertrophy and osteogenesis as indicated by extensive calcification of surrounding matrix (to independently verify these results, we partially repeated expression analysis of matrix molecules throughout differentiation, as reported in one of our earlier studies²³). Thus far, whole embryos or the growth plate are the models of choice for study of endochondral bone formation in vivo^{5,6,20}. This novel concept represents a relevant pre-clinical animal model, which thus enables us to study periosteum derived endochondral bone formation in vivo in the context of physiologically relevant signaling. Cartilage is known to lack vascularization, and oxygenation probably decreases during this first step of PC formation⁹. In addition, because of metabolic activity and concomitant oxygen consumption, oxygen tension is expected to be low in the direct vicinity of cells³⁰. These factors combined most likely contribute to reduced oxygen tension in our model system. The increase in mRNA expression levels of GAPDH and HIF-1 α and the presence of HIF-1 α protein are indicative of a response to a change in oxygenation, and a metabolic shift indicative of adaptation to hypoxia. Under normoxia, the HIF-1 α subunit is rapidly degraded via a concerted action of prolyl hydroxylases, which hydroxylate HIF-1 α , and the von Hippel-Lindau tumor suppressor gene product VHL, which ubiquitylates HIF-1 α and thereby targets it for proteasomal degradation. Oxygen deprivation blocks hydroxylation and thereby stabilizes HIF-1 α . These processes play an important role in chondrocyte growth arrest and survival under hypoxia^{31,31}. During the chondrogenic phase, many essential factors are activated by HIF-1 α -dependent mechanisms^{8,14,33-35}. HIF-1 α plays an important role in the “Pasteur effect” which includes decreased oxidative phosphorylation and an increase in glycolysis³⁶. Since glycolytic ATP production is significantly less efficient per molecule of glucose compared to that by oxidative phosphorylation, glycolytic metabolism is up-regulated to sustain free ATP levels in the hypoxic cell. In good agreement with published data in other model systems, our PC formation model shows a concomitant increase of HIF-1 α and GAPDH mRNA levels, one of the key enzymes in glycolysis and a known HIF-1 α target³⁶. Interestingly, while the mRNA expression levels of BMP-2, -4, and -7 were significantly decreased during chondrogenesis, these growth factors were readily detectable by immunohistochemical staining at 10 and 40 days PO. Reports on BMP expression levels during chondrogenesis are inconsistent: Yaoita et al. report an unaffected expression of BMPs after inducing a fracture, while Kloen et al. report a positive staining of BMPs in human

callus³⁷⁻³⁸. Others report a rapid upregulation of BMP-2, only 24 h after surgical release of the periosteum³⁹. Uusitalo et al. reported an inducing effect on periosteal endochondral callus formation in mice upon adenovirus-mediated gene delivery of BMP-2⁷. The downregulation of BMP-2 at the RNA level we observe is part of a genetic reprogramming controlled by autologous regulatory sequences, which may be absent in adenoviral vectors. Factors like these most likely contribute to fine-tuning of the in vivo response and further substantiate the complexity of BMP-2 regulation during chondrogenesis. The results of the study presented herein indicate that, throughout periosteal callus formation and osteochondrogenic differentiation, BMP expression is regulated both by transcriptional and post-transcriptional mechanisms. Increased association with polysomes during conditions of cell stress (e.g. hypoxia) has recently been shown to result in preferential translation of select mRNAs⁴⁰. It is tempting to speculate that such mechanisms act on BMP and other mRNAs. The exact mechanism of translational regulation of cartilage matrix molecules, BMPs, Sox9, Ihh, PTHrP, and periostin during chondrogenesis under conditions of lowered oxygen availability remains unknown and is subject of further study in our laboratories. PTHrP expression was found in periosteum. In the growth plate, this molecule diffuses to sites of hypertrophic differentiation of chondrocytes to inhibit this process^{2,41}. Ihh was not expressed in periosteum, but its expression was markedly increased during chondrogenesis at 10 days, which is an indicator of hypertrophic chondrocyte differentiation⁴¹. Combined, the presence of both Ihh and PTHrP, the sites at which they are co-expressed, and the timing of their expression are highly indicative of a function for the Ihh/PTHrP interplay in periosteal callus formation equivalent to their proposed role in growth plate models⁴¹. In contrast to other reports^{14,19}, in which POSTN mRNA was not detected in chondrocytes, the present study shows that POSTN protein was detectable in mature chondrocytes. POSTN is an extracellular matrix/adhesion molecule¹⁶, however, its exact function during osteo- and chondrogenesis is unclear. Our immunolocalization studies confirmed this notion, in that POSTN is also detected in the cartilaginous matrix around POSTN-positive chondrocytes. POSTN expression, both at the mRNA and protein level, is increased during fracture healing possibly to enhance the recruitment and adhesion of chondro- and osteo-progenitors from essential sources such as bone marrow and blood¹⁹. The higher number of peripherally located POSTN-positive cells in the newly formed cartilage is consistent with this idea. In line with our observation that both TGF- β and POSTN have similar mRNA expression profile, several studies report a TGF- β dependency of POSTN regulation⁴². POSTN expression is regulated downstream of cellular stress responses, including hypoxia, i.e. during vascular remodeling in different systems^{13,42,43}. Ectopic POSTN expression in several models increases adhesion, migration, and invasiveness^{44,45}. These properties may relate to its proposed role in epithelial-mesenchymal transition⁴⁴. As a ligand for α -V/ β -3 and α -V/ β -5, POSTN mediates that growth promoting and angiogenic signaling in experimental cancers^{46,47}. We do not find significant up-regulation of POSTN at the mRNA level, yet protein detection suggests that POSTN may be regulated in a cell type specific manner during chondrogenesis in our model. The combined above

data suggest that POSTN may mediate comparable processes during chondrogenesis and endochondral bone formation. Whether regulation of POSTN includes a selective translational component, for instance in PC, awaits further analysis. In conclusion, in our periosteal callus formation model, the sequential steps of endochondral bone development are recapitulated and we were able to describe these processes in some cellular and molecular detail: presumptive chondrocytes undergo well-ordered and controlled phases of proliferation, hypertrophic differentiation, mineralization of the surrounding matrix, and finally the cartilaginous tissue is replaced by bone. We have provided evidence that HIF-1 α is activated during the chondrogenic phase of periosteal callus formation, which suggests that conditions during callus formation and differentiation are, at least transiently, hypoxic. Interestingly, expression of BMPs appears to be regulated at a posttranscriptional level during these processes.

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Supplemental Tables

Table 1
Semi-quantitative RT-PCR primer sets

cDNA	Size	Primer sequence		GenBank accession	Homology with human
COLI	511	5'-GCACCCATGGACATTGGAGGG-3'	S	AY633663	92%
		5'-GACACGGAGTAGCACCATCG-3'	AS		
COLX	329	5'-ACAGGAATGCCTGTGTCTGCTTTT-3'	S	AY598937	91%
		5'-TTGGGAAGCTGGAGCCACACCTGGT-3'	AS		
TGF- β_1	271	5'-CGGCAGCTGTACATTGACTT-3'	S	AF000133	nc
		5'-AGCGCACGATCATGTTGGAC-3'	AS		
Sox9	553	5'-TGAATCTCCTGGACCCCTTC-3'	S	AY598935	96%
		5'-CCGTTCTTCACCGACTTCCT-3'	AS		
Runx2	280	5'-TGATGACACTGCCACCTGTG-3'	S	AY598934	99%
		5'-ACTCTGGCTTTGGGAAGAGC-3'	AS		
POSTN	293	5'-CCACGAGGTGCTCCTAGAAAGG-3'	S	AY598936	91%
		5'-TGTTTTCCAGCCAGCTCAAT-3'	AS		
Ihh	318	5'-TTCCAGGTCATCGAGACTCA-3'	S	AY598933	90%
		5'-CCAGAAGGCCAACTGAGC-3'	AS		
28S rRNA	188	5'-GGGTTTAGACCGTCGTGAGA-3'	S	AF460236	nc
		5'-GCGGGATTCTGACTTAGAGG-3'	AS		

Table 2
Real time RT PCR primer sets

cDNA	Size	Primer sequence		GenBank accession
COLI	81	5'-CTGACTGGAAGAGCGGAGAGTAC-3'	S	AY633663
		5'-CCATGTCGCGAGAAGACCTTGA-3'	AS	
COLII	84	5'-TGGGTGTCTATTATTATTGTCTTCCT-3'	S	S83370
		5'-GCGTTGGACTCACACCAGTTAGT-3'	AS	
BMP-2	76	5'-AGAAAAGCGTCAAGCGAAACA-3'	S	AF041421
		5'-GTCCACGTACAAAGGGTGTCTCT-3'	AS	
BMP-4	66	5'-ACCCGACGCTAGCAAGA-3'	S	AF042497
		5'-CTGACTGGAGCCGGTAAAGATC-3'	AS	
BMP-7	79	5'-GCAACCACCTGGGTGGTCAAC-3'	S	AF413111
		5'-TGATGCTCTGCCATCCA-3'	AS	
Sox9	79	5'-AGTACCCGACCTGCACAAC-3'	S	AY598935
		5'-CGCTTCTCGCTCTCGTTCAG-3'	AS	
Runx2	70	5'-TGATGACACTGCCACCTCTGA-3'	S	AY598934
		5'-GCACCTGCCTGGCTCTTCT-3'	AS	
POSTN	87	5'-AATACTCTCCAGTGTCTGAGGCTATT-3'	S	AY598936
		5'-CAGCTTATCTCAATTGTGTTTCTT-3'	AS	
HIF-1 α	79	5'-GGATATAAATTGGCAATGCTCCAT-3'	S	AY273790
		5'-GAGTGCAGGGTCAGCACTACTTC-3'	AS	
VEGF	73	5'-ACGAAGTGGTGAAGTTCATGGAA-3'	S	AY196796
		5'-AAGATGTCCACCAAGGTCTCGAT-3'	AS	
GAPDH	107	5'-ACTTTGTGAAGCTCATTTCTGGTA-3'	S	L 23961
		5'-GTGGTTTGAGGGCTCTTACTCCTT-3'	AS	
28S rRNA	73	5'-GCCATGGTAATCCTGCTCAGTAC-3'	S	AF 460236
		5'-GCTCCTCAGCCAAGCACATAC-3'	AS	

Abbreviations: AS, antisense; S, sense.

Chapter 4

The immediate early gene EGR1 controls epigenetic reprogramming through Polycomb Group proteins during chondrogenesis.

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In preparation

Abstract

Initiation of and progression through chondrogenesis is driven by changes in the cellular microenvironment. For instance, Insulin Receptor signaling is known to mobilize resting mesenchymal stem cells and to initiate a complex, step-wise chondrogenic differentiation program *in vivo* and *in vitro*. Differentiation requires coordinated transcriptomic reprofiling and increased progenitor proliferation, both processes require chromatin remodeling. The nature of early molecular responses that relay differentiation signals to chromatin is poorly understood. We here show that immediate early genes are rapidly and transiently induced in response to a changed cellular microenvironment. Functional ablation of Egr1 severely affects induction of genes required for proper differentiation. In addition, differentiating cells accumulate DNA damage, activate a DNA damage response and induce a senescence-like response, which terminates in global histone hypermethylation. Novel molecular connections with Polycomb Group function identified in this report begin to explain the replicative arrest. We here for the first time describe an important role for Egr1 in early epigenomic reprogramming, exceeding its classical role as transcription factor, to accommodate early gene-environment interactions in chondrogenesis.

Introduction

Differentiation requires orchestration of numerous parallel cellular responses, processes and altered physiological states associated with the novel cell fate. Such changes are often induced by environmental cues (*i.a.* soluble factors, cell-cell contacts), which are signaled to the nucleus and translated into spatio-temporal re-profiling of gene expression. As coding and non-coding genes are 'packed' in chromatin, access to and activation of genes during differentiation is subject to important epigenetic regulation, including covalent modifications to histone proteins and chromatin structure remodeling. Post-translational modifications on histone tails serve to recruit either transcriptional activators or repressors and nucleosome remodeling machineries, and as such constitute an epigenetic register of expression potential ¹⁻³. Recent studies have provided important insights in epigenomic changes that accompany lineage commitment of ES cells. ES cells respond rapidly to changes in the micro environment: as ES cells are induced to differentiate, their epigenome is remodeled such that lineage-related genetic programs are selectively activated, whereas non-lineage relevant genes are progressively inactivated. Part of these epigenetic alterations includes post-translational modifications at amino-terminal tails of histones, which are closely associated with DNA, *i.e.* genes, as they are the major constituents of nucleosomes. Interestingly, many key developmental regulator genes in pluripotent ES cells appear bivalently marked: they carry both 'activating' (*e.g.* H3K4me₃; histone H3, lysine 4, trimethyl) as well as 'inactivating' (*e.g.* H3K27me₃) epigenetic marks in the chromatin surrounding transcription start sites. This bivalent marking switches to monovalency as the differentiation program proceeds ⁴. These observations suggest that although common epigenetic principles exist to initiate cell fate decisions, specific cellular cues are integrated that eventually allow successful lineage demarcation.

Microenvironmental factors play an important role in chondrogenesis. As chondrogenic progenitors move away from the growth plate, their microenvironment changes constantly. These changes include soluble growth and differentiation factors, hormones, oxygen tension, cell-cell and cell ECM contacts ⁵⁻⁸. Cells respond to these changes in the microenvironment by altering their gene expression profiles, which in turn are accompanied by locally changing in the epigenome. An important open question concerning the most primary choices that are made by undifferentiated cells is: What connects these environmental cues to chromatin? Which signaling factors are involved in early epigenomic remodeling and, hence, in differentiation? The Polycomb repressive Complex (PRC) proteins Ezh2 and Bmi1 are important factors in cell fate determination in that they provide cells with an epigenetic memory function: Ezh2 is a histone lysine methyl transferase (HKMT) in Polycomb repressive complex 2 (PRC2), which writes the H3K27me₃ mark ⁹. Bmi1 is part of Polycomb repressive complex 1 (PRC1), which is recruited to H3K27me₃ marks and maintains transcriptionally repressed states ¹⁰⁻¹². As such, PRCs are recognized to sustain lineage commitment in the context of development. We and others have recently shown that Polycomb

function is regulated by post-translational modifications (reviewed by HN JWV; *in press*), suggesting a link between cell signaling and epigenetic re-programming involving PRC. The observation that PRC LOF mutant mice often display abnormal skeletogenesis indicates a potential link to endochondral ossification (*i.e.* formation of an ossified skeleton from a cartilagenous scaffold)¹³⁻¹⁶.

We here study chondrogenesis as a function of environmental changes. On their way to becoming terminally differentiated chondrocytes, chondrogenic progenitor cells *in vivo* undergo a well-described sequential series of events at the cell biology level: initially resting growth plate stem cells undergo a transit replicative burst. *In vivo*, this rapid progenitor expansion in the so called proliferative zone is a distinctive feature of developing cartilage. During endochondral ossification, as cells move away from the growth plate, they differentiate, become hypertrophic and are replaced by bone tissue^{17, 18}. Chondrogenesis is controlled by numerous well-described environmental and endocrinoid factors^{6-8, 17-19}. Per example, signaling through the insulin receptor has been intensively studied because of its mobilizing effect on resting stem cells and stimulatory effects on cells in the proliferative zone²⁰. ATDC5 cells were originally identified as stem cells of mesenchymal origin that harbor chondrogenic potential²¹. The ATDC5 model uniquely combines a number of relevant elements. Chondrogenic differentiation of ATDC5 cells reiterates numerous micro-environmental changes that accompany dynamic and strictly timed transcriptomic re-profiling observed during embryogenesis²¹⁻²³. In addition, proliferation is rapidly upregulated early in differentiation²¹. Hence ATDC5 cells represent an excellent model to study gene-environment interaction at the epigenetic level.

The rapid activation of *immediate early genes* (IEG) denotes the first line of cellular responses to environmental stimuli. Expression of IEG encoded proteins precedes *de novo* synthesis of other proteins; as such IEGs represent an important gateway to genomic responses and physiological adaptation. For instance, *FOS*, *JUN* and *EGR1* (*KROX24/NGFR1*) induction is a well-described feature of neurotransmission^{24, 25}. In the context of cancer, *EGR1* acts either oncogenic or tumorsuppressive²⁶, hence, in some cancers it is overexpressed, in others *egr1* is deleted.

Not much is known about the role of *EGR1* in chondrogenesis. We here report that IEG family members (*i.a.* cFos, cJun, EGR) are rapidly induced in response to insulin signaling. We show that *egr1* mRNA induction in chondrogenesis is transient and precedes transcriptional upregulation of *SOX9*, a known key regulator of chondrogenesis. Acute loss of *Egr1* prevents *SOX9* induction, supporting a role for *Egr1* in transcription of *SOX9*. In addition, loss of *Egr1* leads to replication arrest in culture, which correlates with increased *TRP53* and *P21*^{CIPWAF} expression. In line with the above, loss of *Egr1* has long-lasting effects on epigenomic reprogramming. Finally, we here describe a novel biological association between *Egr1* and Polycomb Group function, providing at least a partial explanation for the observed defective differentiation in the *Egr1*-deficient model.

Materials and methods

Cell culture

ATDC5 cells were cultured at 37 °C, 5% CO₂, 100% humidity in DMEM/ F-12 supplemented with 5% fetal calf serum (FCS), antibiotics (100 units/ml penicillin and 100 µg/ml streptomycin), 200 mM L-glutamine on tissue culture plates (Greiner Bio-One). For differentiation experiments cells were seeded at 6400 cells/cm² and were allowed to attach overnight. Growth medium was changed for differentiation medium, which includes ITS (10 µg/ml insulin, 10 µg/ml transferrin and 3 x10⁻⁸M sodium selenite). Differentiation medium was replaced every two days.

Retroviral infections

Ecotropic retroviral supernatants were produced by transfection of phoenix cells with calcium-phosphate precipitation; 24-48 hours post-transfection, the supernatant was harvested, filtered and used for infection of ATDC5 cells in presence of 4µg/ml polybrene. Cells were incubated for 12 hours and then allowed to recover for 24 hours on fresh medium before selection pressure was applied. Infected cells were selected with puromycin (8 µg/ml) for 72 hours, before experiments were initiated. Short-hairpin (sh)RNA target sequence for Egr1: 5'- ACAAAGTAACCTGTTTGGC-3'. Short hairpin sequence targeting shGFP was used as reference sh sequence [44].

Chrystal violet

ATDC5 cells were plated in 12-multiwell plates (Greiner Bio-one). At each time point cells were washed twice with phosphate-buffered saline, and fixed for 10 minutes with 3.7% formaldehyde at room temperature. Next, cells were rinsed 5 times with demiwater. Cells were stained with 0.1% X-tal violet for 30 minutes or overnight, respectively, and washed 5 times with demiwater. X-tal was extracted with 10% acetic acid and absorbance was measured spectrophotometrically at 590 nm (Benchmark, Biorad).

RNA isolation and cDNA synthesis

Cells were suspended in Tri-reagent, and RNA was extracted according to the protocol of the manufacturer (Sigma). Quantity and purity of the RNA were determined by 260/280 nm absorbance measurements. cDNA was synthesized using iScriptTM cDNA synthesis kit (Biorad). Primers used are listed in supplemental Table 1.

Western analysis

Cells were lysed in RIPA buffer containing 5 mM Benzamidine, 5 µg/ml Antipain, 5 µg/ml Leupeptin, 5 µg/ml Aprotinin, 1 mM Sodium Vanadate, 10 mM Sodium Fluoride, 10 mM Pyrophosphate, 10 mM β-glycerophosphate, 0.5 mM DTT and 1mM PMSF. Lysates were subjected to 3 freeze-thaw cycles in liquid nitrogen, and to 3 cycles of sonication. After centrifugation for 10 minutes at 13.2 krpm, protein concentration was determined using a BCA

protein assay kit (Pierce). Equal amounts of protein were boiled in Laemmli buffer and loaded on 9-15% polyacrylamide gels. Proteins were transferred to polyvinylidene fluoride (PVDF) membranes. After blocking with 5% non-fat dry milk in PBS containing 0.1% Tween-20, membranes were incubated o/n at 4°C with the following antibodies: anti β -actin Mab (C4, 691001, MP Biomedicals), anti Gapdh, anti Ezh2 Mab (BD43; courtesy D. Pasin Copenhagen, Denmark), anti Bmi1 Mab (F6; Courtesy M. van Lohuizen; Amsterdam, The Netherlands), anti Egr1 Poab sc-110 (Santa Cruz Biotechnology), anti Chk1-DCS-310 (Abcam Ab22610), anti p-Chk2 (Thr68) #2661 (Cell signaling), anti γ H2A.X (Ser139) Poab #2577 (Cell signaling), anti TRP53 Mab, anti geminin (FL-209) Poab (Santa Cruz Biotechnology), anti H3K4me3 Poab (ab8580) (Abcam), anti H3K27me3 Poab, #07-449 (Upstate), anti Kap1 Poab A300-275A (Bethyl Laboratories, anti H3K9/14Ac Poab (#06-599, Upstate Biotechnology). After extensive washing, membranes were incubated with corresponding horseradish peroxidase conjugated secondary antibodies for 1h at room temperature. Signals were detected using enhanced chemoluminescence.

Immunofluorescence

Briefly, cells were washed and fixed for 10-15 minutes in 100% methanol at -20°C and stored at 4°C in 70% ethanol or used directly for immuno-histochemistry. Cells were permeabilized for 5-10 minutes in 0.2% triton-X in PBS. To prevent epitope loss in combination with acid treatment for BrdU detection, primary antisera were incubated for 1.5 hours at 37°C, 4-5x washed in 0.02% triton-X/PBS and fixed in 2% formaldehyde/PBS for 10 minutes at room temperature. Cells were washed with PBS and incubated for 20 minutes in 2.0N HCl at 37°C, followed by two rinses of 0.1 M sodium tetraborate solution, pH 8.5 for in total 2 minutes. Cells were then incubated with the primary antisera against BrdU, washed at indicated before and incubated with secondary fluorescently labeled antibodies. All Antisera were diluted and incubated in 0.02% triton-X in PBS. Nuclei were counterstained with 4'-6-Diamidino-2-phenylindole (DAPI) and washed in 0.02% triton-X/PBS. The last wash step was in PBS, upon which cells were dehydrated: 1 minute in 70% ethanol, 2x 1 minute in 100% ethanol and air-dried. Cells were mounted in Vectashield (Vector Laboratories, Inc. Burlingame, CA) and analyzed using a NIKON TE200 Eclipse fluorescence microscope and photographed using a NIKON DXM1200 digital camera in combination with NIS Elements 3.0 Imaging software. The following antibodies were used: anti γ H2A.X Mab JBW301 (Upstate), anti BrdU (BD biosciences) and anti H3K9me3 #07-442 (Upstate), anti H3K27me3 Poab, #07-449 (Upstate).

Chromatin Immunoprecipitation

ChIPs were performed and analyzed essentially as described previously⁵¹.

Briefly, U373 and HCT116 cells were fixed in 1% formaldehyde. Cross-linking was allowed to proceed for 10 min at room temperature and stopped by addition of glycine at a final concentration of 0.125 M, followed by an additional incubation for 5 min. Fixed cells were washed twice with PBS and harvested in SDS Buffer (50 mM Tris at pH 8.1, 0.5% SDS, 100 mM NaCl, 5 mM EDTA), supplemented with protease inhibitors (Aprotinin, Antipain and Leupeptin all at 5 µg/mL and 1 mM PMSF). Cells were pelleted by centrifugation, and suspended in IP Buffer (100 mM Tris at pH 8.6, 100 mM NaCl, 0.3% SDS, 1.7% Triton X-100, and 5 mM EDTA), containing protease inhibitors. Cells were disrupted by sonication, yielding genomic DNA fragments with a bulk size of 200-500 bp. For each immunoprecipitation, 1 mL of lysate was precleared by addition of 35 µL of blocked protein A beads (50% slurry protein A-Sepharose, Amersham; 0.5 mg/mL fatty acid-free BSA, Sigma; and 0.2 mg/mL herring sperm DNA in TE), followed by clarification by centrifugation. 10 µL aliquots of precleared suspension were reserved as input DNA and kept at 4°C. Samples were immunoprecipitated overnight at 4°C with antibodies for either HA as a negative control (sc-805; Santa Cruz), H3K27me3 (07-449; Upstate), anti H3K4me3 Poab (ab8580) (Abcam), anti Egr1 Poab sc-110 (Santa Cruz Biotechnology). Immune complexes were recovered by adding 40 µL of blocked protein A beads and incubated for 4 h at 4°C. Beads were washed three times in 1 mL of Mixed Micelle Buffer (20 mM Tris at pH 8.1, 150 mM NaCl, 5 mM EDTA, 5% w/v sucrose, 1% Triton X-100, and 0.2% SDS), twice in 1 mL of Buffer 500 (50 mM HEPES at pH 7.5, 0.1% w/v deoxycholic acid, 1% Triton X-100, and 1 mM EDTA), twice in 1 mL of LiCl Detergent Wash Buffer (10 mM Tris at pH 8.0, 0.5% deoxycholic acid, 0.5% NP-40, 250 mM LiCl, and 1 mM EDTA), and once in 1 mL of TE. Immuno-complexes were eluted from beads in 250 µL elution buffer (1% SDS; and 0.1M NaHCO₃) for 2 h at 65 °C with continuous shaking at 1000 rpm, and after centrifugation supernatants were collected. 250 µL elution buffer was added to input DNA samples and these were processed in parallel with eluted samples. Crosslinks were reversed overnight at 65°C followed by a 2h digestion with RNAse A at 37°C and 2h proteinase K (0.2 µg/µL) at 55°C. DNA fragments were recovered using QIAquick PCR purification columns, according to manufacturers' instructions. Samples were eluted in 75 µL EB buffer and then further 1/5 diluted in TE buffer. The immunoprecipitated DNA was quantified by real-time qPCR. Primers used are listed in supplemental Table 1.

Affymetrix gene arrays

Affymetrix NuGO mouse arrays (~24k) were run on RNA from ATDC5 cultures sampled over time post induction of differentiation (*pid*). For each time point 3 samples were taken from both a wildtype (WT) and a Egr1 knock-down (KD) culture. After scanning, data preprocessing and analysis were done using R2.7.1 with the use of Bioconductor libraries^{80,81}. Data were ²log transformed and normalized using gcRMA⁸².

Results

Egr1 is rapidly induced in differentiation

To identify early responses to differentiation stimuli, ATDC5 cells were stimulated to undergo chondrogenesis. An expression-array experiment was designed to assay immediate early, early and late changes in gene expression profiles, as a function of time (0, 2, 4, 8, 16, 24 and 72 hrs). Expression analysis revealed a rapid upregulation of a number of immediate early growth response genes (IEGs): expression of IEGs belonging to the *FOS*, *JUN* and *EGR1* subfamilies is significantly enhanced within 2 hours upon induction of chondrogenesis in murine mesenchymal progenitor ATDC5 cultures (Suppl. Figure 1A). Also c-MYC is induced, albeit at more moderate levels (2x; FS, GvdA, JWV; *unpublished data*).

Figure 1. Egr1 is rapidly induced in chondrogenic differentiation and binds to chondrogenic promoters.

(A) qRT rtPCR analysis of Egr1 expression up to 4 hours *pid* with 20 or 30 minute intervals. (B) qRT rtPCR analysis of Sox9 expression up to 4 hours *pid* with 20 or 30 minute intervals. (C, E, G) ChIP using anti-Egr1 antibodies and n; 0, 2 and 8 hours *pid* immunoprecipitated DNA was analyzed by qRT rtPCR for the respective promoters. (D,F,H) ChIP using anti-H3K27me3 and anti-H3K4me3 antibodies and n; 0, 2 and 8 hours *pid* immunoprecipitated DNA was analyzed by qRT rtPCR for the respective promoters.

Expression of most IEGs rapidly declines over the next 2 hours. To investigate a role for IEGs in chondrogenesis we focused on EGRs; *EGR1* and *EGR3* are both massively induced upon adding ITS: *EGR1* expression is induced up to 200x at the mRNA and returns to baseline levels within 4 hours *pid* (post-induction of differentiation). An independent real time expression analysis using a time resolution of 20 minute intervals essentially confirmed the microarray findings: *EGR1* mRNA increases within 20 minutes into differentiation, peaks at 1-2 hours and falls off to pre-induction levels within 2-3 hours (Figure 1A). *EGR1* expression increases again later during chondrogenesis around 6 days (Suppl Figure 1B). Protein expression parallels the observed changes in *EGR1* mRNA levels nicely: whereas absent at t=0, Egr1 protein is first detected at 1 hour *pid*, peaks at 2 hours and rapidly declines thereafter (Figure 2A).

Egr1 initiates chondrogenesis through SOX9 and RUNX2 expression

This early IEG-induction profile suggested a role for EGR1 in activating downstream differentiation programs. To chart potential target genes for Egr1 and to build a chondrogenic gene-interaction network, we combined published data, with a mouse genome-wide screen for Egr1 binding sites and software tools to predict promoter binding sites for numerous transcription factors. Multiple consensus binding sequences for Egr family members exist²⁷⁻²⁹ we confined our genomic scan to GCGG/TGGGCG motifs and its reverse complementary sequence, both in sense and antisense orientation. Genes containing predicted binding sites were mapped using pathway analysis tools (Pathvisio with Wikipathways content; Suppl Figure 2). Several known key-regulators of chondrogenesis, such as *SOX9* and *RUNX2*³⁰⁻³³, contain predicted Egr1 binding sites. At the mRNA level, *SOX9* shows a transient, approximately 1 hour out-of-phase induction in response to differentiation medium as compared to *EGR1* (Figure 1B). *In silico* *SOX9* promoter analysis using GENOMATIX software revealed several putative binding sites for Egr family members a number of which correspond to potential Egr1 consensus binding sites (Suppl Figure 3). To confirm the *in silico* mapping, we performed chromatin immunoprecipitation (ChIP) with anti-Egr1 antibodies on ATDC5 cells differentiated for 0, 2 and 8 hours. Two independent primer sets were designed to detect co-precipitated *SOX9* promoter sequences using real time PCR. In good agreement with the expression kinetics of *EGR1* upon induction of differentiation, both *SOX9* primer sets show substantial enrichment of Egr1 within the vicinity of the Egr1 binding sites at 2 hours *pid* (10-15 fold; Figure 1C; Suppl Figure 4A). Likewise, the *RUNX2* promoter is enriched 67-fold for Egr1 at 2 hrs (Figure 1E). Both promoters carry histone H3K4me3, an epigenetic status permissive for transcriptional activity (Figure 1D, F; Suppl Figure 4B). Consistent with a sharp drop in protein

levels (Figure 2A), 8 hours into differentiation, Egr1 occupation at these promoters was restored to levels found in undifferentiated cells. Conversely, the Aggrecan (*ACAN*) promoter, appears not enriched for Egr1 at 2 hours *pid*, and, in contrast to the *SOX9* and *RUNX2* promoters, *ACAN* is marked for repression with H3K27me3 (Figure 1G, H). Both findings are consistent with a significantly later transcriptional activation of *ACAN* in chondrogenesis as opposed to *SOX9* and *RUNX2* at approximately 10 days (Suppl Figure 4C-H). To firmly establish a role for Egr1 in chondrogenesis, we studied chondrogenic marker gene expression in the context of RNA-interference mediated knock-down (KD) of EGR1. A shRNA vector targeting murine *EGR1* mRNA (shEGR1) was designed based on criteria published before³⁴. Expression analysis at both mRNA level and protein level shows that the retroviral shEGR1 vector efficiently targets *EGR1* expression (Figure 2A,B). Both *SOX9* and *RUNX2* display a multi-phasic expression profile throughout differentiation (Suppl Figure 4C-F). Relevantly, loss of *EGR1* function (*EGR1*-LOF) blunts early *SOX9* and *RUNX2* expression (Figure 2C; Suppl Figure 4D). Egr1 enrichment in the *SOX6* promoter is only marginally changed and *EGR1*-LOF appears to have very little effect on mRNA expression (Suppl Figure 5A,D).

Figure 2. Egr1 is required for induction of chondrogenic differentiation.

(A) Western blot analysis of Egr1 expression in ATDC5 cells stably expressing shControl or shEgr1 at 0, 1, 2 and 4 hours *pid*. GAPDH is used as loading control (B) qRT rtPCR analysis of Egr1 expression in ATDC5 cells stably expressing shControl or shEgr1 up to 4 hours *pid*. (C) qRT rtPCR analysis of Sox9 expression in ATDC5 cells stably expressing shControl or shEgr1 up to 12 hours *pid*. (D,F) qRT rtPCR analysis of collagen 2a1 and aggrecan expression in ATDC5 cells stably expressing shControl or shEgr1 up to 15 days *pid*. (E) Western blot analysis of chondrogenic marker expression in ATDC5 cells stably expressing shControl or shEgr1 up to 15 days *pid*.

This suggests that *SOX6* and *SOX4* loci may not be prime Egr1 targets for transcriptional activation early in chondrogenesis; indeed both *SOX6* and *SOX4* are not expressed until later in chondrogenesis (Suppl Figure 5C). Expression of genes encoding the ECM proteins Collagen type II (*Col2A1*) and Acan is activated 7-10 days *pid* in reference cultures; Egr1-KD significantly reduces *COL2A1* and *ACAN* expression at the mRNA and protein level (Figure 2D,E; Suppl Figure 4C). *RUNX2*-induced Collagen type X (*COL10A1*) expression coincides with hypertrophic differentiation³⁵; *Col10A1* levels are severely reduced in Egr1-KD cultures (Figure 2E). Thus, activation of Egr1 early in chondrogenesis directly activates transcription of key regulators of downstream differentiation pathways.

Loss of Egr1 elicits replication stress induced senescence-like cell cycle arrest.

An important feature of early chondrogenesis is the capacity of chondrocytes to rapidly proliferate. We studied ATDC5 differentiation induced hyperproliferation as a function of *EGR1* expression. Proliferation rate of control cells is approximately 3 fold increased from 1 day *pid* onward as compared to cells under normal, non-differentiating culture conditions (Figure 3A). Differentiating cells typically overgrow each other and form nodules, *i.e* focal points of chondrogenesis (Suppl Figure 6). The proliferative burst coincides with a second period of *EGR1* upregulation (Suppl Figure 1B), indicating a potential requirement for *EGR1* during enhanced cell division. Remarkably, *EGR1*-LOF completely abrogates the ability of ATDC5 cells to hyperproliferate and cells cease proliferation when they reach confluency (Figure 3B; Suppl Figure 6). The proliferative block was independently confirmed using incorporation of BrdU as a read-out (Figure 3C). The hyperproliferative block is accompanied by dramatic morphological changes: *EGR1*-LOF cells display the 'large flat cell' phenotype typically associated with cellular senescence (Figure 3D). Consistent with this, ATDC5 *EGR1*-LOF cultures show polyploidy and reveal transcriptional induction of numerous genes implicated in cellular senescence, DNA damage and stress signaling (Suppl Figure 7).

Figure 3. Loss of Egr1 elicits replication stress induced senescence-like cell cycle arrest.

(A) ATDC5 cells hyperproliferate upon induction of chondrogenic differentiation. Proliferation determined by Crystal violet incorporation with subsequent extraction and spectrophotometric analysis. (B) ATDC5 cells stably expressing shEgr1 fail to undergo differentiation induced hyperproliferation, measured by Crystal violet incorporation. (C) ATDC5 cells stably expressing shEgr1 or shControl were differentiated for 2 days, then BrdU was administered to the cells for 45 minutes. BrdU was visualized with anti-BrdU antibodies. (D) Morphological analysis of ATDC5 cells stably expressing shEgr1 or shControl at 4 days *pid*.

Activation of a DNA damage response (DDR) was confirmed by immunoblotting of phosphorylated H2A.X (γ H2A.X) and Chk2 in chondrogenic cultures (Figure 4A-B). In control cells Chk2 is phosphorylated at a basal level and slightly reduced during hyperproliferation, differentiation in the absence of Egr1 induces a substantial increase in pChk2 at day 3 (Figure 4A). Upregulation of

several cell stress markers suggests that the Atm/Atr DDR (pChk2, γ H2A.X; Chk1) pathway and cell cycle arrest (Trp53, Gmnn) are activated. Consistent with this, immunohistochemical staining for γ H2A.X confirms DNA damage in Egr1-KD cells under differentiating conditions (Figure 4C). To map processes regulated by Egr1, the genes identified in a genome-wide blast for Egr1 binding sites were analyzed using GenMAPP; this yielded a number of overrepresented processes known to be involved in chondrogenesis and differentiation in general (table 1).

Figure 4. Egr1-LOF elicits DNA damage in transit amplifying chondrocytes

A, B) Western blot analysis of proteins related to DNA damage responses in ATDC5 cells stably expressing shControl or shEgr1. bActin is used as loading control. (C) Protein expression of γ H2Ax in ATDC5 shControl and shEgr1, at 2 days pid. Counterstaining by DAPI.

In addition, we identified cytokine signalling pathways as potential targets of Egr1. Relevantly, interleukins, like IL6, are known to play an important role in establishment of senescence^{36, 37}. In good agreement with this, IL6 is significantly induced upon differentiation in the absence of Egr1 (Suppl Figure 7). Taken together, this data clearly identifies Egr1 as an essential regulator protein for hyperproliferation in the context of chondrogenesis and shows loss of Egr1 results in cellular senescence-like response.

Egr1 controls epigenomic remodeling

As Egr1 controls both transcriptional reprogramming and hyperproliferation, we studied global epigenomic changes throughout chondrogenesis as a function of Egr1. Histone acetylation (H3K9/K14ac and H4K12ac), trimethylation at lysine 27 (H3K27me3), lysine 4 (H3K4me3) and lysine 9 (H3K9me3) all undergo dynamic changes throughout chondrogenesis: all trimethyl marks peak during hyperproliferation and gradually increase toward hypertrophy (Figure 5A). In contrast, histone acetylation is high in *shcon* chromatin at the initial stages of chondrogenesis, and gradually declines over time (Figure 5A). Remarkably, all epigenomic changes were substantially perturbed in Egr1-KD cultures. Consistent with a role in recruiting HAT (histone acetyl transferase) activity, *EGR1*-LOF cells display a striking inability to induce and/or maintain acetylation levels throughout hyperproliferation (Figure 5B). In contrast, trimethylation levels at histone H3K4, H3K9 and H3K27 appear initially low and substantial elevate at later time points in the absence of Egr1 (Figure 5B, C). We next investigated the effect of Egr1-KD on a number of H3K27me3 and K9me3-associated proteins. Polycomb Group proteins are known to affect skeletogenesis *in vivo*. The Polycomb PRC2 and PRC1 complex are both functionally connected to the H3K27me3 mark. Egr1-KD cultures fail to properly induce BMI1 both at the mRNA and protein level, suggesting a direct effect of EGR1 on BMI1 expression (Figure 5D; Suppl Figure 8A). Also Ezh2 expression is deregulated in Egr1-KD cultures: under normal conditions Ezh2 protein levels slightly increases in response to early signaling in chondrogenesis; this induction fails in Egr1-KD cultures (Figure 5E; Suppl Figure 8A). Conversely, Ezh2 is substantially induced in Egr1-KD cells at late time points in differentiation (Figure 5E). The enhanced Ezh2 expression correlates well with the increase in H3K27me3 (Figure 5C,G).

The presence of multiple predicted EGR-family member consensus binding sites in the Bmi1 promoter, and a more than 50 fold increased Egr1 presence at the Bmi1 promoter (Suppl. Figure 8C, D) are consistent with direct control of Bmi1 expression by Egr1. The Ezh2 promoter is substantially less enriched for Egr1 (± 4 fold) and Ezh2 mRNA levels are not consistently suppressed in the absence of Egr1 arguing for a possibly secondary effect of EGR1-LOF on expression of Ezh2. (Suppl Figure 8B,E). A substantial increase of KAP1/TIF1B expression, a factor associated with HP1/H3K9me3, correlated well with the observed increase in H3K9me3 levels at late time points in Egr1 LOF cultures (Figure 4C,F,G). In summary, the above data demonstrate that Egr1 is crucial for initiation of an epigenome wide reprogramming during chondrogenesis and that differentiation in the absence of Egr1 results in abnormal epigenetic responses.

Figure 5. Loss of Egr1 affects histone modifications and histone modifiers.

(A,B,C) Western blot analysis of histone modifications in ATDC5 shControl and shEgr1 throughout chondrogenesis. (D,E,F) Western blot analysis of PcG proteins and Kap1, a novel PcG interacting protein, in ATDC5 cells stably expressing shControl or shEgr1. (G) Immunocytochemical analysis histone modifications in ATDC5 cells stably expressing shControl or shEgr1.

Egr1-promoter binding is not sufficient for transcriptional activation

The genome-wide abundance of Egr1-family consensus binding sites and of Egr1 in particular, combined with the dramatic effects of Egr1-LOF on global epigenomic reprogramming during chondrogenesis suggested that Egr1 may not enhance transcriptional activity by itself upon DNA binding. The *SOX9* promoter is predicted to be in a relatively 'open' chromatin configuration at t=0, based on the presence of transcriptional activity-associated H3K4me3 marks and the absence of repressive H3K27me3 marks. This chromatin state is maintained early in chondrogenesis (Figure 1D). The same holds true for *RUNX2*, which, as *SOX9*, is free of repressive H3K27me3 marks (Figure 1F), and for PRC gene promoters of *BMI1* and *EZH2* (Suppl Figure 8F, G). All 'open' chromatin promoters, i.e. only marked H3K4me3, appear enriched for Egr1 at 2 hrs of induction of

differentiation. Yet, not all these “open” genes neither follow the induction profile typical of e.g. *SOX9*, nor lose induction of gene activity in the absence of *Egr1* as *SOX9* does. In contrast, gene promoters of *ACAN* and *SOX6*, appear initially silenced by PRC1 (Figure 1H, Suppl Figure 5B): the *ACAN* promoter carries H3K27me3 marks, consistent with *ACAN* being a PRC1 target in cells of mesenchymal origin ¹¹. Indeed, a comparison of chondrogenic promoters with putative *Egr1*-binding sites and reported Polycomb-associated gene promoters yielded a number of potential common target genes for both *Egr1* and Polycomb (Suppl Figure 2). *SOX6*, a presumed *Sox9* target, gradually loses its PRC-associated repressive mark over the initial 8 hrs in differentiation. Regardless of these initial epigenetic conversions, however, neither *ACAN* nor *SOX6* are transcriptionally activated until late in chondrogenesis. Neither gene is substantially enriched for *Egr1*, despite the presence of predicted *Egr1* binding sites in their promoters (Suppl Figure 3) suggesting that a repressive chromatin confirmation prevents *Egr1* access.

To independently test the hypothesis that *Egr1* binding does not induce gene activity *per se*, we analyzed *HOX* cluster gene expression in the context of *Egr1* presence. *HOX* genes are key developmental regulators of antero-posterior (AP) patterning and skeletal development ^{38,39}. *HOX* genes are functionally grouped in 4 multi-gene clusters in mammals; expression within these clusters is subject to co-linear regulation, hence expression boundaries within a cluster are sharply defined in a cell-context specific manner ^{40, 41}. *HOX* clusters carry predicted *Egr1* binding sites (Suppl Figure 9B).

To examine induction of *HOX* gene activity in relation to *Egr1* enrichment, *HOXA* cluster genes (*A1*, *A4*, *A6*, *A7*, *A9* and *A11*) were subjected to ChIP analysis for *Egr1*, H3K27me3 and H3K4me3 during early chondrogenic differentiation. Relevantly, a gradient of epigenetic marking over the *HoxA* cluster is readily visible: *HOXA1* and *HOXA4* promoters are up to 400-fold enriched for H3K4me3, whereas *HOXA6* shows a lower, approximately 20-fold enrichment compared to the HA-control (Figure 6A). None of these *HOXA* genes are enriched for H3K27me3. The *HOXA7* promoter appears only moderately enriched for both epigenetic modifications, whereas *HOXA9* and *HOXA11* lack H3K4me3, they display enrichment for the repressive H3K27me3 mark (Figure 6A). These findings predict an expression boundary within the *HOXA* cluster at *HOXA6/A7* in non-differentiated ATDC5 cells. Strikingly, *Egr1* is only enriched at epigenetically accessible *HOXA* promoters, as defined by H3K4me3-marking; none of the H3K27me3 promoters are enriched (Figure 6B). Despite the increased *Egr1* occupation at 2 hours *pid*, *Egr1*-binding does not induce transcriptional regulation of any gene within the *HOXA* cluster, irrespective of epigenetic status (Figure 6C). Consistent with this, none of the *Hox* gene clusters respond to *EGR1*-LOF (Suppl Figure 9). Taken together the above data shows that *Egr1* binding of consensus sequence motifs is not sufficient for activation of transcription.

Figure 6. Egr1-promoter binding is not sufficient for transcriptional activation. (A,B) ChIP analysis for enrichment of H3K27me3, H3K4me3 and Egr1 on promoters of the HoxA cluster. (C) Relative mRNA expression of the HoxA cluster in ATDC5 cells differentiated for up to 24 hours.

Discussion

We here report for the first time that many IEGs are rapidly induced in the context of chondrogenesis: Egr1 is highly induced and controls relevant chondrogenic pathways: RNA interference-mediated knock-down of Egr1 affects early global chromatin acetylation and severely impedes chondrogenesis: both expression of key regulator genes of chondrogenesis, like SOX9 and RUNX2, as well as transit amplification are blocked. The block in rapid progenitor expansion correlates with DNA-damage induced signaling and display characteristics of a senescence-like response. Our analysis also shows that Egr1 promoter binding does not activate transcription *per se*, and suggests a more general role for Egr1 in epigenomic reprogramming during differentiation.

The observation that Egr1-KD specifically blocks rapid differentiation-induced progenitor expansion strongly suggests that replication stress is involved in the proliferative block. The conditions at onset of differentiation show remarkable resemblance to oncogenic stress and several lines of evidence suggest parallels with oncogene induced senescence (OIS). Firstly, ATDC5 cells undergo a transit proliferative amplification, a situation identical to the early increase in proliferation induced by oncogenes ⁴²⁻⁴⁴. Secondly Egr1-KD cells accumulate DNA damage and activate a DDR, as evidenced by phosphorylation of H2AX, Chk2 and Kap1, and upregulation of multiple DDR genes. Thirdly, in the context OIS, cell cycle exit and irreversible senescence are dependent on activation of a DDR ⁴³. We show here that DDR in Egr1-KD cells coincides with a strong replicative block and a senescence-like phenotype: cells display distinctive features associated with senescence: large flat cell morphology, polyploidy, expression of numerous senescence-associated marker genes. Combined this data strongly argues that Egr1 facilitates proliferative expansion in transit amplifying chondrogenic progenitors.

The early abnormal global acetylation in the absence of Egr1 is in good agreement with Egr1's ability to recruit HAT activity ⁴⁵. C-Myc, one of the first IEGs identified, was recently found to augment global acetylation through both transcriptional regulation and recruitment of GCN5 ⁴⁶. Early acetylation may be required for making rapid proliferation possible. It is conceivable that global acetylation in response to DNA binding of IEG-encoded proteins may help define regions within a network of increased DNA-templated activity, and support differentiation-specific changes in transcription and proliferation.

It is currently only possible to speculate about the biological relevance of the co-regulation of seemingly conflicting trimethyl levels on H3K4, H3K9 and H3K27 under normal and stressed conditions. It seems unlikely, given their stability across cell generations, that these changes in global trimethylation reflect a cell cycle dependent enrichment. Besides with transcriptional activity, each mark is associated with distinctive other processes. H3K9me3 is a hallmark of heterochromatin. H3K9me3 and HP1 accumulation at distinct nuclear foci has been associated with replication stress induced senescence ⁴⁷. Increased accumulation of H3K9me3 at these senescence-associated heterochromatin foci (SAHF) is the consequence of subnuclear redistribution rather than increased global H3K9me3 levels ⁴⁸. We identified several indicators of a senescence-like response in Egr1-KD cultures *pid*. In addition, however, a global H3K9me3 increase at later time points (9, 15 days) is observed. The elevated H3K9me3 is preceded by increased KAP1 expression, which was reported to increase heterochromatin formation through recruitment of SETDB1 ⁴⁹. KAP1 is specifically required to resolve DNA damage in heterochromatin, down stream of ATM signaling ⁵⁰. As Kap1 and HP1 physically interact ⁵¹, the elevated global H3K9me3 levels may point to increased heterochromatin formation. DNA damage in senescent cells was reported to persist for weeks on end ⁵². As such H3K9me3 and Kap1 may provide a means to protect damaged DNA from replication.

Ezh2 levels were found to decrease in the context of replicative senescence⁵³. Consistent with this, Ezh2 levels decrease during replicative stress in Egr1-KD cultures at 6 days *pid*. At late time points, elevated H3K27me3 correlates with increased Ezh2 expression. It is conceivable, that the formation of trimethylated chromatin represents a protective response against abnormal differentiation, cell death or transformation. Of note: all three trimethyl marks on histone 3 increase during transit amplification, suggesting a role under conditions of increased chromatin stress. In line with this notion, increased H3K4me3 and H3K27me3 have both been associated with other cellular stress responses: we and others have observed increased histone 3 K4me3 and K27me3 in response to hypoxia or conditions that evoke replication stress (⁵⁴,FS, PP and JWV; *unpublished data*). In addition, H3K4me3 was identified as a crucial determinant of Rag2-mediated V(D)J recombination in B and T cells, and is linked to DDR-induced cellular responses via ING family proteins^{55, 56}. These combined observations suggest roles for global trimethyl marking in processes in addition to local regulation of transcription.

Interestingly we observe potential mechanistic interactions of Egr1 with PRC function at several levels. Firstly, loss of Egr1 affects, directly or indirectly, expression of two important PRC members, Ezh2 and Bmi1. Both proteins have been associated with cell cycle regulation: Ezh2 is a direct target of E2F1⁵⁷, and Bmi1 controls expression of P16/INK4A and P14^{ARF}, both encoded by the CDKN2/INK4A locus^{58, 59}. Secondly, comparative analysis of published PRC targets and a genome wide scan for Egr1 consensus binding sites yielded potential common targets and suggests coordinated regulation. Indeed we observe a close correlation between the presence of PRC-repressive H3K27me3 marks and lack of Egr1 enrichment 2 hours *pid*, suggesting that PRCs prevent Egr1 access to promoters. Interestingly, likewise, H3K27me3 prevents Myc from interacting with its genomic targets⁶⁰. Importantly, we recently found that PRC1-LOF has comparable effects on chondrogenesis as Egr1-LOF, suggesting that at least part of the Egr1-LOF phenotype can be explained by PRC-LOF (FS, JWV: *in preparation*).

Finally, Egr1 was reported to be sumoylated in a P14^{ARF} mediated process⁶¹. The responsible E3 ligase, UBC9, also interacts with PRC1 & 2^{62, 63}. Egr1-sumoylation is required for transcriptional activation of the Egr1-target PTEN. The protein phosphatase PTEN deactivates PI3K, which in turn will no longer activate AKT. In our experimental setting, however, we observe no induction of PTEN mRNA and Egr1-KD only marginally affects PTEN expression. This would suggest, that either most regulation occurs at the level of post-translational modification or that the EGR1-PTEN-AKT-INK4AARF axis may not be a prime regulatory module early in chondrogenesis. In line with cell context dependent responses, several studies report cell type-dependent responses an Egr1-targeted reporter gene was activated in neural cells, whereas in cartilage and heart it was suppressed^{64, 65}.

Epigenomic reprogramming by Egr1 may serve to define concerted transcriptional or replication activity of gene-networks to guide cells through chondrogenesis. In agreement with such a coordinating role, Egr1 is known to transactivate and recruit CBP/P300, the consequence of which

is increased local HAT activity⁶⁶. In contrast, Egr1 interaction with NAB1/2 and recruitment of the NuRD complex would repress transcription by bringing HDAC activity to target genes⁶⁷⁻⁶⁹. The global distribution of Egr1 binding sites may point to a more general task in epigenomic reprogramming, not exclusively linked to transcription. By analogy, recent studies on genomic distribution of transcription factor (TF) binding sites (PPAR γ , ER α) identified up to half of such binding sites either in intragenic regions (introns) or at distant locations (>25 kb away from the nearest gene), suggest additional epigenomic roles besides TF binding in gene promoters⁷⁰⁻⁷². It is tempting to hypothesize a role for IEGs in early epigenomic pre-programming, such that ensuing processes (concurrent transcription and replication) are facilitated in the context of development. Several observations support a close relationship between epigenetic marking and changed DNA replication during differentiation. Replication of lineage-relevant and non-relevant genes appears functionally separated in time: relevant genes ('open' chromatin^{73, 74}) is replicated earlier in S-phase than non-lineage related genes. Dynamic change of replication timing throughout differentiation is crucial, and regulation of these processes is provided by the micro-environment of a stem cell or progenitor. Although still far from completely understood, translation of this epigenomic 'landscape'⁷⁵ provides the 'traffic signs and road maps' to terminally differentiated cell fates. It will be of considerable interest to map global and local acetylation in respect to Egr1 binding and replication activity. Likewise, correlation of K4, K9 and K27 trimethyl marks, and likely numerous additional epigenetic marks, in the ATDC5 Egr1-KD system to DNA-templated activity will help advance insight into epigenetic regulation of the genome. These experiments are currently underway.

Although epigenetic mechanisms in differentiation begin to emerge, exact knowledge of how cells communicate environmental changes to chromatin and how global epigenomic remodeling accompanies differentiation is lacking. At the onset of the study reported here, knowledge on IEGs in the context of chondrogenesis was limited. A general loss of Egr1 expression was reported in degenerating human cartilage⁷⁶. Although EGR family members may regulate each others expression⁷⁷ several studies provide evidence for distinctive functions for individual Egr1 family members. In adipogenesis, Egr1 and Egr2 have different roles⁷⁸. In keeping with this, the here reported strong phenotypic response to Egr1-LOF, despite induction of other closely related Egr-family IEGs, point to absence of functional compensation and argue for a unique role for EGR1 in early chondrogenesis: the acute nature of an RNAi mediated knock-down approach used in this study, may prevent activation of redundant mechanisms often observed in null-mutant models⁷⁹. Here, we report a dual function for the transcription factor Egr1 in activating a lineage-specific transcriptional re-programming, as well as transit progenitor expansion. The inappropriate chromatin reprogramming in the absence of Egr1 strongly support an important task for Egr1 in early epigenomic remodeling during chondrogenesis and pave the way for further studies in gene-environment interactions.

Table 1: Genmapp overrepresentation table (database used: Mm_Derby_20090509.pgdb; processes with a Z score > 1.96 are presented).

Pathway	positive (r)	measured (n)	total	%	Z Score
Insulin Signaling	46	156	160	29,49%	5,10
EGFR1 Signaling Pathway	48	171	172	28,07%	4,83
IL-4 signaling Pathway	21	58	60	36,21%	4,50
Regulation of Actin Cytoskeleton	37	146	150	25,34%	3,50
G Protein Signaling Pathways	24	84	87	28,57%	3,47
IL-6 signaling Pathway	26	96	97	27,08%	3,30
Wnt Signaling Pathway NetPath	28	107	109	26,17%	3,22
Calcium Regulation in the Cardiac Cell	36	148	151	24,32%	3,17
IL-2 Signaling Pathway	20	73	74	27,40%	2,94
IL-5 Signaling Pathway	18	65	66	27,69%	2,84
miR-1 in cardiac development	3	5	5	60,00%	2,80
IL-3 Signaling Pathway	24	95	97	25,26%	2,78
Kit Receptor Signaling Pathway	17	65	66	26,15%	2,49
Heme Biosynthesis	4	9	9	44,44%	2,45
MAPK Cascade	9	29	30	31,03%	2,39
Nuclear Receptors	11	38	38	28,95%	2,38
Mouse ESC Pluripotency Pathways	25	109	112	22,94%	2,30
Proteasome Degradation	19	78	81	24,36%	2,29
Circadian Exercice	13	49	49	26,53%	2,23
Circadian Exercice	13	49	49	26,53%	2,23
Id Signaling Pathway	13	49	51	26,53%	2,23
Myometrial Relaxation and Contraction	34	160	161	21,25%	2,20
MAPK signaling pathway	29	134	138	21,64%	2,13
Wnt Signaling Pathway	15	61	62	24,59%	2,07
IL-9 Signaling Pathway	7	23	24	30,43%	2,05
IL-1 Signaling Pathway	10	37	38	27,03%	2,02
IL-7 Signaling Pathway	11	42	44	26,19%	2,00
p38 MAPK Signaling Pathway (BioCarta)	8	28	30	28,57%	1,98

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Figure S1. Expression profiles Immediate Early Genes throughout chondrogenesis.

(A,B) Microarray analysis of Immediate Early Genes mRNA expression in ATDC5 chondrogenic differentiation presented as fold induction compared to T=0 (A), or arbitrary units (B).

Figure S2. Enchondral ossification pathway analysis for predicted Egr1 targets and published PcG targets (Bracken et al.)

A genome-wide blast search was performed for Egr1 consensus binding sequences. The search was confined to GCGG/TGGGCG motifs and its reverse complementary sequence, both in sense and antisense orientation. Genes containing predicted binding sites were mapped using pathway analysis tools (Pathvisio with Wikipathways content).

Figure S3. Genes involved in chondrogenesis contain multiple Egr1 consensus binding sites. Using GENOMATIX promoter analysis software, promoters were analyzed for Egr1 binding sites. Arrows indicate primers designed for qRT rtPCR to quantify immunoprecipitated chromatin.

Figure S4. Egr1 directly binds and regulates the Sox9 promoter. (A,B) Chromatin immunoprecipitation using anti-Egr1, anti-H3K27me3 and anti-H3K4me3 antibodies. Precipitated DNA was analyzed by gRT rtPCR for enrichment on the Sox9 promoter using primer set 2 (see figure S3). (C-H) Microarray mRNA expression analysis of chondrogenic genes in ATDC5 cells stably expressing shControl or shEgr1 throughout chondrogenesis.

Figure S5. Egr1 directly binds and regulates the Sox6 promoter

(A,B) Chromatin immunoprecipitation using anti-Egr1, anti-H3K27me3 and anti-H3K4me3 antibodies. Precipitated DNA was analyzed by qRT-PCR for enrichment on the Sox6 promoter. (C-F) Microarray mRNA expression analysis of Sox6 and Sox4 in ATDC5 cells stably expressing shControl or shEgr1 throughout chondrogenesis.

Figure S6. Loss of Egr1 affects differentiation induced hyperproliferation
Morphology analysis of ATDC5 stably expressing shControl or shEgr1 during the first 6 days of chondrogenesis.

Figure S7. Loss of Egr1 induces numerous genes implicated in cellular senescence, DNA damage and stress signaling. Microarray mRNA expression analysis of genes involved in cellular senescence, DNA damage and stress signalling in ATDC5 cells stably expressing shControl or shEgr1 throughout chondrogenesis.

Figure S8. Egr1 directly binds PcG promoters and regulates PcG expression.

(A,B) Relative mRNA expression of *Bmi1* and *Ezh2* in ATDC5 stably expressing shControl and shEgr1 up to 4 days *pid*. (C) using GENOMATIX promoter analysis software, *Bmi1* and *Ezh2* promoters were analyzed for Egr1 binding sites. Arrows indicate primers designed for qRT rPCR to quantify immunoprecipitated chromatin. (D-G) Chromatin immunoprecipitation using anti-Egr1, anti-H3K27me3 and anti-H3K4me3 antibodies. Precipitated DNA was analyzed by gRT rPCR for enrichment on the indicated promoter.

Figure S9. Egr1 promoter binding is not sufficient for transcriptional activation.

(A) Microarray mRNA expression analysis of all Hox clusters in ATDC5 cells stably expressing shControl or shEgr1 throughout chondrogenesis. (B) The number of predicted Egr1 binding sites (GENOMATIX) in the promoters of the respective Hox genes.

Chapter 4

Supplemental Table 1:

Primers used for qRT rtPCR

Ezh2	Forward	GAAAAAAGATGAGACGTCCAGCTC
	Reverse	GAGGTTCAATATTTGGCTTCATCTTTAT
AggreCAN	Forward	CATGAGAGAGGCGAATGGAA
	Reverse	TGATCTCGTAGCGATCTTTCTTCT
Sox9	Forward	AGTACCCGCACCTGCACAAC
	Reverse	TACTTGTAGTCCGGGTGGTCTTTC
Bmi1	Forward	TGGAGAAGAATGGCCCACT
	Reverse	GCAAGTTGGCCGAACCTCTGT
Collagen 2a	Forward	CAAGAACCCTGCTCGCACTT
	Reverse	CCGCTCTTCCACTCGGG
Egr1	Forward	AGCCGAGCGAACAACCCTAT
	Reverse	TGTCAGAAAAGGACTCTGTGGTCA

Primers used for Chromatin Immunoprecipitation

Sox9-1	Forward	GTTCCAAAAGTGTGACATTCCG
	Reverse	TCCGCGTGTGTAAGTTTGCTCTCT
Sox9-2	Forward	TCTCCTGGACCCCTTCATGA
	Reverse	GCCAGACAGGCCCTTCT
Sox6	Forward	AAGCCAATTGTCTTTGTCAGTT
	Reverse	ATCTTTGAAACAGAGAAATTGACAGTG
Hoxa1	Forward	TCACTGAGTGATTGGATCCTGC
	Reverse	GGAGGAAGTGAGAAAGTTGGCAC
Hoxa2	Forward	GACAAGGTTGAAATTGGACCG
	Reverse	CAAATTGTCATTGGGCAGAAGC
Hoxa4	Forward	CTCTGGAATAAAACGAAGGAGGC
	Reverse	GGACAAAGAATCAAAGGGCGAG
Hoxa6	Forward	CTTTCCTTTTTTGCCTTCATGG
	Reverse	TTGTCAGGTTTCCTGTTTGGG
Hoxa7	Forward	AACCCTTCCCCTAAACGCCTC
	Reverse	AAAAGGTCGCCAGTCTTCCAG
Hoxa9	Forward	ATCTGTATGCCTAGTCCCGCTCC
	Reverse	TTGATGTTGACTGGCGATTTTC
Hoxa11	Forward	GGAAGCAACAGATCGTCACTCG
	Reverse	TGAGTTACACCGGCGATTACG
Ezh2	Forward	GAGGCGCTTGATAGTGCTGG
	Reverse	ACCCAATCGCCATCGCT
Bmi1	Forward	CATTTTGGAGCCGGTGTCA
	Reverse	AAAAAATGCACGGCTGAAGG

Polycomb Repressive Complex 1 Coordinates Enhanced Replication and Transcription in Transit Amplifying Chondrogenic Progenitors.

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In preparation.

Abstract

Segmentation phenotypes in Polycomb loss-of-function mutants correlate with defective maintenance of *HOX* gene expression boundaries along the antero-posterior axis, yet little is known about the possible direct involvement of Polycomb Repressive Complex 1 proteins (PRC1) in enchondral ossification. We here investigate chondrogenesis in relation to loss of Polycomb function. We provide evidence that normal chondrogenic hypertrophy *in vitro* and *in vivo* shares characteristics with replicative senescence. We find that acute shRNA-mediated reduction of PRC1-function compromises transit amplification during differentiation: rapid accumulation of massive DNA damage and activation of an intra-S-phase checkpoint result in a senescence-associated secretory phenotype and a senescence-like replicative arrest. Using global nuclear analysis of active transcription and replication, we show that coordinated regulation of active transcription and replication is lost in Bmi1-deficient cells and that DNA damage occurs at sites of DNA synthesis. Hence, acute loss of PRC1-function during early replication in chondrogenesis renders cells unable to deal with increased chromatin stress. This is the first report revealing an important function for PRC1 in orchestrating simultaneous chromatin-associated processes, i.e. DNA replication and transcription damage repair, in differentiating transit amplifying cells.

Polycomb Group proteins act in transcriptionally repressive complexes (Polycomb Repressive Complexes; PRC), which vary in protein composition between cell types ¹. These multi-component PRCs maintain gene expression profiles in the context of differentiation and development. PRC-mediated silencing is controlled at the chromatin level by epigenomic indexing: PRC complexes install and bind post-translational modifications on N-terminal histone tails, often in conjunction with associated epigenetic activity ^{2,3}. Well-known PRC-associated epigenetic marks are H3K27me3 and H2AK119ub1, both are required for PRC-mediated transcriptional repression ²⁻⁵. As such PRC are believed to constitute a molecular epigenetic memory and fulfill an important task in maintaining cell identity.

Analogous to PRC mutations in *Drosophila Melanogaster*, which cause abnormal segment identity development along the antero-posterior (AP) axis, the effect of PRC1 loss-of-function (LOF) on murine skeletal development correlates with defective maintenance of expression boundaries in *HOX* gene clusters ⁶⁻⁹. The exact role of PRC1 in bone and cartilage formation however, is not clear. Moreover, the relatively mild skeletal phenotypes of numerous single PRC1-null mutants as opposed to compound PRC1 mutants ⁹⁻¹³, suggest that redundant mechanisms may mask acute effects of loss-of-PRC1 function during skeletogenesis. The mature ossified mammalian skeleton develops from a cartilaginous scaffold (endochondral bone formation); we therefore studied chondrogenesis *in vitro* in the context of PRC1 loss-of-function (LOF). The murine ATDC5 embryonal mesenchymal stem cell line reiterates the multi-step chondrogenic differentiation program observed during endochondral bone formation and is an excellent, widely used *in vitro* model system to study chondrogenesis ¹⁰: a growth factor-induced differentiation program is initiated which is readily monitored by chondrogenic marker gene expression (e.g. *SOX9*, *RUNX2*, *Collagen type II* and *X* (*COL2A1*, *COL10A1*), *Aggrecan* (*ACAN*); ¹¹. We focused on PRC1 complex genes with known null-mutant skeletal phenotypes ^{7,12-14}. Several PRC1 complex genes are highly expressed, among which *BMI1* (*PCGF4*), *PHC1* and *2* (*EDR1/MPH1/RAE28* and *EDR2/MPH2*) and *RNF2* (*RING1B*), and regulated throughout chondrogenesis (figure S1A): Bmi1 protein expression is elevated at 6 days (figure 1A, B) post induction of differentiation (*pid*), pointing to an increased reliance on Bmi1 function. Retroviral short hairpin RNAs (shRNA) were designed to target both human and mouse (sh*BMI*hm) or exclusively human (sh*BMI*h) *BMI1* mRNA for RNAi-mediated degradation ((figure 1B,C; S1B). RNAi-mediated Bmi1 knock-down (KD) delays chondrogenesis as evidenced by reduced *ACAN* and *COL2A1* expression (figure 1D); conversely sh*BMI*h has no effect on differentiation, in line with its selective targeting of human *BMI1* mRNA (figure S1C). In keeping with published gene dosage effects on skeletal phenotypes in PRC1 genetically modified mice ^{6,8,12,13}, re-expression of *mBMI1* cDNA in ATDC5-sh*BMI*hm cells restores Bmi1 expression and induces the exact opposite effect of sh*BMI*hm on chondrogenic marker gene expression (figure 1E, 1SD). Thus this data demonstrates the significance of the ATDC5 model for probing PRC1-function, and is consistent with a specific function for PRC1 in chondrogenesis.

Figure 1. Bmi1 expression is required for chondrogenic differentiation.

(A) qRT rtPCR analysis of Bmi1 expression during ATDC5 chondrogenesis. (B) Western blot analysis of Bmi1 protein expression in ATDC5 cells stably expressing shControl or shBmi1 (control versus differentiation medium) at t=0 and 6 days. (C) Immunofluorescence analysis of Bmi1 expression in ATDC5 shControl and shBmi1 and DAPI nuclear staining. (D) qRT rtPCR analysis of chondrogenic markers in ATDC5 shControl and shBmi1 up to 15 days *pid*. (E) qRT rtPCR analysis of ACAN in Bmi1 overexpressing ATDC5 and control cells up to 15 days *pid*.

Numerous published data support a role for PRC1 proteins, including Bmi1, in cell cycle regulation under normal and oncogenic conditions¹⁷⁻²⁰. The exact mechanism(s) by which PRC control cell cycle in different settings, however, are insufficiently clear. Differentiating ATDC5 monolayer cultures typically become multi-layered and develop distinctive three-dimensional *nodules* that produce cartilage matrix proteins¹⁰. In line with a reduced chondrogenic potential, nodule-formation is delayed and reduced in numbers in Bmi1-KD cultures (see below: figure 4C). As multi-layering and nodule formation are, at least in part, dependent on sustained proliferation, we examined cell proliferation in relation to PRC1-LOF. Under non-differentiating conditions, Bmi1-KD marginally affects cell proliferation (figure 2A,B). Upon induction of differentiation, proliferation rate in control cultures increases substantially from 1 day *pid* (fig 2A) and is sustained until 5-6 days

pid; this early, rapid, transient increase in cell division, is observed in various cell systems and resembles transit amplification (TA) of progenitor cells^{15,16}. Relevantly, transient rapid progenitor proliferation is part of normal endochondral ossification *in vivo*, and is apparent in the proliferative zone of growing long bones (fig S2A). Remarkably, Bmi1-KD cells fail to undergo transit amplification *pid*: cell proliferation does not exceed that of cells (Bmi1-KD or control) under continuous culture conditions (figure 2B). Of note: the proliferative burst of differentiating ATDC5 cells corresponds in time with elevated Bmi1 expression (figure 1A,B), suggesting transit amplification is either controlled by or controlling Bmi1 levels. Transit amplification responds positively to Bmi1 dosage: overexpression of Bmi1 increases proliferation slightly yet significantly (figure S2A). Under non-differentiating conditions, DNA profiling and BrdU incorporation assays reveal neither significant differences in cell cycle phase distribution nor in BrdU incorporation (figure 2C, S2B) between Bmi1-KD and control cells. In sharp contrast, differentiating Bmi1-KD cells show a substantially increased S-phase population at 6 days *pid* (figure S2B). Similarly Phc2-KD reduces cell proliferation *pid* and increases S-phase accumulation, pointing to a general involvement of PRC1 in transit amplification (figure S2C). Transient BrdU exposure at 6 days *pid* reveals active incorporation of BrdU by rapidly dividing control cells and confirmed a block in *de novo* DNA synthesis in Bmi1-KD cultures (figure 2C). Consistent with this, differentiating ATDC5 Bmi1-KD cultures display significantly less G2/M related H3S10 phosphorylation (figure 2D). Importantly, normal healthy human cartilage-derived chondrogenic precursors respond similarly to BMI1-LOF: proliferative and chondrogenic capacity are severely reduced (figure 2E). Combined, the above data support the notion that cells lacking PRC1 cannot replicate their DNA as efficiently as control cells during the hyperproliferative phase. An intra-S-phase arrest is indicative of stalled replication forks; these are known to coincide with double strand DNA breaks (DSB) and locally induce Ser139 phosphorylation on histone H2AX (γ H2A.X). Under normal (non-differentiation) culturing conditions, ATDC5 cultures (independent of genotype) typically include some cells carrying γ H2A.X foci, presumably due to local replication-associated DSB (figure S3A,B). Normally such DNA damage is rapidly repaired to ensure cell cycle completion¹⁷. At 6 days *pid*, however, γ H2A.X detection in Bmi1-KD cultures is dramatically increased compared to control cells (figure 3A). Differences in DSB are first detectable at 1-2 days *pid* (figure 3B); at 10 days *pid* DNA damage is substantially reduced (figure 3C); control cultures do not accumulate as much damage, yet show detectable γ H2A.X throughout the proliferative phase (figure 3C). Differentiating Bmi1-deficient primary human h929 cells also show elevated γ H2A.X (figure 3D). High-density culturing of chondrogenic models triggers differentiation even in the absence of ITS. Consistent with differentiation-induced DSB, high-density Bmi1-KD cultures show S-phase accumulation and increased DNA damage as opposed to control cultures (Figure S3C). Whereas Phc2-LOF has comparable effects on progenitor expansion under these conditions (figure S3D), interestingly, the effect of PRC1-LOF on chondrogenic potential differs between PRC1 members (figure S3E).

Figure 2. Loss of Bmi1 elicits replication stress induced S-phase arrest.

(A) ATDC5 cells hyperproliferate upon induction of chondrogenic differentiation, as compared to continuous culture conditions (cc). Proliferation determined by Crystal violet incorporation with subsequent extraction and spectrophotometric analysis. (B) ATDC5 cells stably expressing shBmi1 fail to undergo differentiation induced hyperproliferation, measured by Crystal violet incorporation. (C) ATDC5 cells stably expressing shEgr1 or shControl were differentiated for 6 days, then BrdU was administered to the cells for 45 minutes. BrdU was visualized with anti-BrdU antibodies and subsequently analyzed by flowcytometry. DNA content is determined by propidium iodide staining. (D) ATDC5 shBmi1 or shControl were differentiated for 3 days, then BrdU was administered to the cells for 45 minutes. BrdU was visualized by immunofluorescence with anti-BrdU antibodies. (E) Human primary chondrogenic precursors stably expressing shBmi1 or shControl were differentiated for 2 days, then BrdU was administered to the cells for 45 minutes. BrdU was visualized by immunofluorescence with anti-BrdU antibodies.

This phenotypic variation may reflect divergent effects of PRC1-LOF on skeletal morphogenesis in full genetic knock-outs *in vivo*^{6,14}. In line with a proliferative block, detection of nuclear γ H2A.X and H3S10ph inversely correlate between control and KD cells at 3 days *pid* (figure S4A). Expression array analysis at 6 days *pid* confirms massive upregulation of DNA damage repair pathways in Bmi1-KD cultures (Table S1); consistent with activation of a DNA damage response (DDR) Bmi1-KD nuclei show ATM-induced Kap1S824 and Chk2-phosphorylation besides enhanced Rad51 and RPA70 chromatin-association (Figure S4B-D; and data not shown).

Figure 3. Replicating shBmi1 expression ATDC5 cells accumulate DNA damage during differentiation induced transit amplification.

(A) Immunofluorescence analysis of DNA damage in ATDC5 shControl and shBmi1 visualized with anti- γ H2A.X antibodies at 6 days *pid*. DAPI used as nuclear staining. (B) Immunofluorescence analysis of DNA damage and replication in ATDC5 shControl and shBmi1 visualized with anti- γ H2A.X and anti-BrdU antibodies, respectively, at 1 day *pid*. DAPI used as nuclear staining. (C) Western blot analysis of γ H2A.X in ATDC5 shControl and shBmi1 throughout differentiation. B-actin and Gapdh as loading controls. (D) Immunofluorescence analysis of DNA damage in human primary chondrogenic progenitors expressing shControl and shBmi1 visualized with anti- γ H2A.X antibodies at 2 days *pid*. DAPI used as nuclear staining.

Loss of PRC1-function causes premature replicative senescence in mouse embryo-fibroblasts cultures and other cell types¹⁸⁻²⁰. Analogously, Bmi1-deficiency induces a cell senescence-like morphology as does LOF of other PRC1-proteins (figure 4A). The presence of γ H2A.X in enlarged cells at 6 days *pid* suggests that these cells probably accrued DSB and activated a DDR and an associated senescence-like response (SLR) overtime (figure 3A), in agreement with the causal relationship between DNA damage and senescence^{21,22}. In line with this notion, also sporadic large flat cells in non-differentiating cultures carry DNA damage and are polyploid irrespective of genotype; large flat Bmi1-KD cells again show substantially more DSB (figure S5A). The relative resistance of ATDC5 under normal growth conditions to Bmi1-LOF was unexpected given its otherwise significant anti-proliferative effect in cell cultures^{26,27,23}. This prompted us to investigate

senescence-related signaling in the context of differentiation. Of note: important checkpoints (*i.a.* contact inhibition, IR induced arrest/senescence; data not shown) are intact in non-differentiating ATDC5. Senescent fibroblasts typically express senescence-associated β -Galactosidase (SAbGal/Glb1)²⁰. Surprisingly, *GLB1* mRNA is induced approximately 2 fold and SAbGal is activated during chondrogenesis *in vitro* (figure 4C, S5B). Importantly, we observe SAbGal-activation in all primary human and rabbit chondrogenic cultures examined (figure S5C). Rapidly expanding murine ATDC5 and primary human h929 cells display focal H3K9me3, a feature reminiscent of senescence associated heterochromatin foci (SAHF;²⁹) and Bmi1-LOF further augments H3K9me3 levels (figure 4B,D, S5C). Besides a global increase of H3K9me3, H3K27me3 increases during hyperproliferative stress in Bmi1-KD cultures (figure 4C,E). Ezh2 protein is slightly more expressed in Bmi1-KD cells at 6 days *pid* and may contribute to enhanced H3K27me3 levels (figure S5E). However, the H3K27me3 demethylase Jmjd3, which is normally induced 4.2 fold during hyperproliferation, fails to do so in Bmi1-KD 6 days *pid* (figure S5F). Lack of *JMJD3* was recently associated with replicative senescence²⁴; hence it is likely that absence of Jmjd3 is responsible for the senescence-associated increase in H3K27me3. In good agreement with activation of SLR, numerous additional markers associated with cell cycle exit and/or a senescence-associated secretory phenotype (SASP;²⁵) are subject to dynamic regulation in the context of normal differentiation and show an exaggerated response (*i.a.* *IL6*, *CCL5*, *CXCL10*) in Bmi1-LOF cultures during hyperproliferation (figure S5G). Bmi1-LOF does not appear to change the onset of SAbGal activation (figure 4C). Remarkably, although overexpression of oncogenic RasV12 in chondrogenic control cultures enhances onset and intensity of SAbGal activation and, it does not induce replicative arrest and leaves chondrogenic potential fully intact (figure 4C, S5H).

We next explored involvement of known senescence-related checkpoints. PRC1-dependent transcriptional regulation of the *CDKN2A/Ink4A* locus²⁶ links Bmi1 directly to cell cycle regulation, stem cell self-renewal and oncogenesis²⁷⁻³³. We could not detect significant differences in expression of *CDKN2A/INK4A*, *TRP53* and *P21^{CIP1/WAF1}* between control and Bmi1-KD cells during chondrogenesis (figure S5C). In addition, preliminary analyses indicate that inactivation of *INK4A/ARF* or *TRP53* does not bypass cell cycle arrest in ATDC5 Bmi1-KD *pid* cells, but instead reveals a dependency of chondrogenesis on these cell cycle regulators (FS, JWV unpublished observations). These findings are in agreement with published reports showing that the intra-S-checkpoint, although pRb and/or Trp53-dependent, is not induced by p16^{INK4A} or P21^{CIP1/WAF}³⁸³⁴. In support of this, a differentiation and S-phase arrest by *CHAF1A/CAF1b*-LOF in zebrafish was not rescued by *Trp53*-LOF³⁵. Our observation in ATDC5 contrasts with a recently uncovered lineage-specific role of p21^{CIP1/WAF1} in NSC self-renewal³⁶, and suggests lineage specific PRC-independent checkpoint mechanisms are operative during self-renewal and/or transit amplification in chondrogenic. Taken together, above observations suggest that the chondrogenic lineage is inherently refractory to signaling known to induce irreversible replicative arrest in other systems.

Figure 4. PRC1-LOF results in replicative senescence; terminal chondrogenic differentiation resembles senescence.

(A) Morphological analysis ATDC5 shControl, shBmi1, shRnf2 at t=2d *pid*. (B) Western blot analysis of senescence associated histone modifications in ATDC5 shControl and shBmi1 throughout chondrogenesis. (C) Senescence-associated bGalactosidase staining on ATDC5 expressing shControl, shBmi1, vector or Rasv12 cultures under control conditions or differentiation conditions (ITS) for the indicated time points. (D,E) Immunofluorescence analysis of senescence associated histone modifications in ATDC5 shControl and shBmi1 at indicated time points. DAPI used as nuclear staining. (F) Senescence-associated bGalactosidase staining only or in combination with alizarin red on ribcages of WT mice. Brackets highlight the hypertrophic zone.

Chondrogenic differentiation during endochondral ossification terminates in hypertrophy and subsequent tissue replacement (mineralization/ossification) by invading osteoclasts and osteoblasts¹¹. Importantly, RUNX2, an oncogene known to activate SLR, is implicated in hypertrophic differentiation^{37,11}. Based on our findings thusfar, we hypothesized that endochondral differentiation may naturally terminate in a senescence-like phenotype, and thus SAbGal-positive cells should be detectable adjacent to growth plates in elongating bones of newborn mice. In line with activation of SAbGal during chondrogenic hyperproliferation *in vitro*, histological analysis of *pp1* (day 1 *post-partum*) skeletal elements revealed prominent SAbGal activity precisely at the prehypertrophic zone in long bones and intercalated disks (figure 4F, S6A,B). SAbGal positive

zones are positioned proximal to mineralization zones in rib bones (figure 4F). We reasoned that PRC1-LOF may result in increased hypertrophy during endochondral ossification which should be reflected in the width of the HZ. In good agreement with this, *RING1A*-KO pp1 mice, which display a comparatively prominent PRC1-LOF related skeletal phenotype¹⁴, revealed an increased HZ (Figure S6C). Thus, our collective findings corroborate a hitherto unrecognized role for PRC1 in chondrogenic transit proliferative amplification.

Figure 5. Fold change increases during transit amplification.

(A) Pie charts of all reporters fulfilling $p < 0.05$, $FC > 1.1$ up or down, expression $> 2^{\log(100)}$ in either group for time points 0, 1 day and 6 days *pid* respectively (for all time points c.f. Figure S7A). For each time point two percentages are indicated: the first indicates the amount of reporters fulfilling all criteria as compared to the number of reporters above expression limit; the second indicates the amount of reporters fulfilling all criteria as compared to the total number of reporters. (B) Boxplots of WT vs. *Bmi1*-KD logratios for all reporters (regardless of expression level, fold change or significance) for each time point in the experiment. (C) Scatterplot for each timepoint versus each other timepoint of the $2^{\log}$ expression values in WT (left) and *Bmi1*-KD (right) cells, based on the reporters fulfilling $p < 0.05$, absolute $FC > 2$, expression $> 2^{\log(100)}$ in either group, at 1 or 6 days *pid*.

We next investigated the origin of DNA damage. As differentiation requires coordinated regulation of several DNA-templated processes, among which transcription and replication, we analyzed the effect of Bmi1-LOF on global transcriptional responses in the context of differentiation. Contrary to expectation, loss of repressive function produced comparable percentages up or down regulated genes (figure 5A, S7A). Transcriptional re-profiling (*i.e.* number of genes involved and fold changes) intensifies when transit amplification is initiated both in control and Bmi1-LOF cultures; however, differences in log expression prior to and following transit amplification are substantially smaller in control cultures than in Bmi1-LOF cultures, indicating much tighter controlled transcriptome reprogramming in the presence of Bmi1 (figure 5C). Differences in gene expression between control and Bmi-LOF cultures become more prominent between 1 and 6 days *pid*: expression of over one third of genes is more than 1.2 fold enhanced (figure 5B, S7B). Transcriptomic deregulation revealed distinctive clustering (figure 5C, S7C). Profile analysis of the most extreme deregulated genes (*i.e.* ≥ 2 fold changed) reveals a number of different profile clusters: genes that are consistently a) higher or b) lower expressed in Bmi1-KD *versus* control throughout differentiation, c) genes of which transcription is low and not regulated during chondrogenesis in control cells, but that dramatically respond to PRC1-LOF at the time when control cells enter the hyperproliferative state, and vice versa d) genes that are expressed and induced at hyperproliferation in control but fail to do so in Bmi1 KD cells (figure S7D). Numerous of the abnormally highly induced genes in KD cultures, map to differentiation programs and pathways irrelevant to chondrogenesis (figure S7E). A number of genes in category c is related to SLR/SASP (figure S7F; see also figure S5C). In addition, induction of some genes appears inconsistent with chondrogenic differentiation. Per example, γ SCrystalline (CryGS), a major functional component of the lens, is normally exclusively expressed by cell of ectodermal origin (ATDC5 cells are mesodermal derivatives). In line with an aberrant induction of CryGS during chondrogenesis, the crystalline gene cluster was recently identified as PcG target in cells of mesenchymal origin³⁸. The diversity of aberrant response profiles combined with the observation that many genes are down-regulated upon PRC1-LOF suggests that besides a function in (direct) target gene repression, PRC1 fulfills additional tasks that impinge on transcription. We therefore focused on the potential role of transcription and replication in the origin of DDR and consequential S-phase arrest. Typically, BrdU-positive cells co-stain for the DNA polymerase processivity factor PCNA (Proliferating Cell Nuclear Antigen) in control cultures (figure S8B); PCNA-dim cells are either in G1 or G2. Consistent with an intra-S-phase arrest, we find a large percentage of cells PCNA positive, yet BrdU negative (figure S8B). Importantly, all BrdU-positive cells are also positive for γ H2A.X in Bmi1-KD cultures (figure 6A); cells that exclusively show γ H2A.X signals arrested prior to BrdU incubation and, moreover, DNA damage in Bmi1-KD cells perfectly co-localizes with BrdU signals (figure 6A). This strongly supports the idea that damage occurs at sites of active *de novo* DNA synthesis and that lack of PRC1 is causally related to replication induced DNA damage. The

more intense staining for Rpa70 (figure S4A), a protein which prevents single stranded DNA from back-folding during DNA-replication, is in good agreement with replication-induced damage. Indeed, Bmi1-KD cultures are significantly more sensitive to the replication poisons etoposide than control cells under normal culturing conditions (figure S8A) (camptothecin similar results; data not shown).

To study transcriptional status, cells were stained for active Ser5-phosphorylated RNA polymerase 2 (pRpol2). Control cells reveal a cell cycle dependent non-uniform nuclear distribution of active pRpol2: pRpol2-bright cells are generally PCNA-dim (G1/early S), whereas late in S-phase pRpol2-negative nuclei are strongly positive for PCNA; likewise, these cells are DAPI-bright (figure S8C); pRpol2-dim nuclear regions in control cells show BrdU incorporation (figure 6B). This nuclear partitioning of DNA-templated activities indicates coordinated regulation of replication and transcription under normal chondrogenic conditions and is in good agreement with earlier reports on dynamic segregation of mRNA and DNA synthesis throughout S-phase³⁹. Remarkably, the non-uniform distribution of pRpol2 is lost in PCNA-bright PRC1 LOF cells, and, moreover, Bmi1-KD nuclei arrested in S-phase show uniform nuclear staining for both pRpol2 and BrdU (figure 6B; S8C), suggesting that concerted regulation of replication and transcription is lost in the absence of intact PRC1 (figure 6B). In addition, damaged Bmi1-KD cells have reduced pRpol2 levels, suggesting reduced transcription as a functional consequence of global DNA damage (figure 6C). DNA damage as a result of failure to tune replication to transcription, and *vice versa*, is expected to not be restricted to abnormally derepressed transcriptional activity, but instead occurs throughout the genome. The finding that many genes show consistent deregulated transcription, regardless of chondrogenic induction (figure 5C) most likely directly relates to defective transcriptional/replicational coordination and enhanced chromatin stress in PRC1-KD cultures.

Bmi1 and Rnf2, were recently identified as a ubiquitin E3 ligase for Geminin (Gmnn/Gem), a factor important for termination of replication at late S-phase^{40,41}. PRC1-LOF stabilizes Gmnn and interferes with haematopoietic stem cell (HSC) self renewal⁴¹. In good agreement with this, we find that Bmi1-KD cells stain positive for Gmnn (Figure 7A), whereas proliferating control cells generally do not. In line with the observed DDR and SLR, all Gmnn positive Bmi1-KD cells co-stain for γH2A.X, and the Cdt1/Gmnn ratio <1 is indicative for re-replication and polyploidy in Bmi1-KD cells (figure 7B). The enhanced Gmnn chromatin-association shows an inverse correlation with global loss of Bmi1 (figure 1C). We recently observed a positive correlation between PRC-phosphorylation, PRC1/chromatin dissociation and Gmnn stabilization (VvL JWV unpublished observations). In the context of stress signaling, redistribution of Phc1 (Rae28), through which Gmnn is associated with PRC1, may provide a docking point for Gmnn stabilization; stress signaling induces chromatin dissociation of other PRC1 members (Bmi1, Ring and CBX proteins; JBC & YT, JWV; unpublished data).

Figure 6. ATDC5 shBmi1 accumulate DNA damage at sites of deregulated transcription and replication. (A,B,C) Immunofluorescence analysis of replication (BrdU), active transcription (pRNA polymerase II) and DNA damage (γ H2A.X) in ATDC5 stably expressing shControl or shBmi1 at 3 days *pid*.

Combining these observations, we propose a molecular switching mechanism that controls local transcription *versus* replication: local presence of Rnf2/Bmi1 represses transcription (H2AK119ub1), targets GMNN for proteolysis, relieving a potential replication block. Conversely, loss of BMI1/RNF2 stabilizes GMNN and blocks replication and is permissive for transcription (figure 7C).

The most direct interpretation of our data is that PRC1 generates a chromatin state which tunes transcription to DNA replication and prevents DNA-damage during a differentiation-coupled proliferative burst. Polycomb proteins are found at promoters also when genes are not silenced⁴².

Figure 7. Accumulation of DNA damage correlates with stabilization of the replication inhibitor geminin.

(A) Immunofluorescence analysis of Bmi1, Phc1 and Geminin in ATDC5 shControl and shBmi1. (B) Immunofluorescence analysis of γH2A.X and Geminin in ATDC5 shControl and shBmi1. Cdt1/Geminin ratio <1 is indicative for re-replication and polyploidy.

(C) Proposed model: PRC1 regulates a switching mechanism that controls local transcription *versus* replication: local presence of Rnf2/Bmi1 represses transcription (H2AK119ub1), targets geminin for degradation, relieving a potential replication block. Conversely, loss of Rnf2/Bmi1 stabilizes geminin and blocks replication and is permissive for transcription.

A substantial amount of H3K27me3 marked genes is expressed in ES cells; in addition, numerous gene promoters are bivalently marked (*i.e.* with both activation-associated H3K4me3 and repression-associated H3K27me3; ⁴³). Many of these promoters map to important developmental control genes, and bivalency often resolves into monovalency, dictated by their involved in specific lineage commitment programs. Gene expression and replication timing are strongly correlated: actively transcribed genes often replicate early. Both replication timing and transcription change substantially in the context of differentiation in mid S-phase, when replication origins are spatially redefined and replication timing and transcription are re-tuned ⁴⁴. Although a single study into

effects of PRC2-LOF on replication timing within the context of neurogenic differentiation revealed no obvious differences, a potential role for PRC1 in replication timing requires analysis in the context mesenchymal lineage commitment ⁴⁵. In addition to a direct involvement in transcriptional repression, it is conceivable that H3K27me3 and H3K4me3 (respectively PcG and trxG-associated epigenetic marks) functionally partition the genome and help coordinate DNA-templated processes like transcription and replication in the context of development. Indeed, PRC proteins interact with basal factors of the transcription machinery as well as replication factors (reviewed in Chapter 2). A link between PRC-function and DNA replication has been suggested before: Polycomb LOF in *D.melanogaster*, results in replication defect ⁴⁶. Hyperproliferation-associated replication damage and senescence are important features of oncogene induced senescence (OIS) and suggest a functional analogy. Chondrocytes appear to have a remarkable ability to hyper-replicate under conditions unfavorable for cell division (e.g. cartilage is extremely hypoxic). In addition, (pre)hypertrophic chondrocytes excrete vast amounts of ECM proteins that build and support the cartilaginous matrix. These conditions are known to induce endoplasmic reticulum (ER) stress signaling (ERSS) and the unfolded protein response (UPR); both ER stress and UPR are known inducers of OIS in the context of tumorigenesis ⁴⁷. ERSS/UPR and hypertrophic zone expansion are also at the basis for chondrodysplasia in mice and men ^{47,48}. The proposed biological analogy between hypertrophy and SLR is also consistent with a shared role for RUNX proteins in OIS and chondrocyte hypertrophy ^{11,37}. These combined observations suggest that chondrogenic cells tolerate replication stress better than other cells. In agreement, we find that oncogenic RasV12 does not induce a replication arrest; in stead chondrogenic differentiation seems more robust. Indeed robust MAPK signaling is important in chondrogenesis ⁴⁹. PRC-LOF induces an exaggerated SASP/SMS response and blocks proliferation as a result of massive replication damage. Conversely, Bmi1 and RasV12 cooperate in oncogenesis ⁵⁰. Hence, transit amplification during chondrogenesis shares important characteristics of cellular responses to oncogenic activation and emphasizes a pivotal role for PRC1.

The findings reported here may point to a novel aspect of Polycomb biology during skeletogenesis: it is conceivable that proliferation and hence tissue expansion, co-determines positioning along the A-P axis and that premature termination of differentiation contributes to the PcG-phenotypes observed in null-mutants. Presence of SAbGAL in intervertebral disk cartilage possibly implicates a proliferative component in axial positioning. It is possible that abnormal *HOX* gene expression patterns contribute to the PRC1-phenotypes observed in null-mutants, both through direct effects on proliferation and differentiation. Indeed, recognition of a potential role for proliferation in axial patterning is long standing ⁵¹. Combined our data suggest that PRC1 plays an important role in DNA damage avoidance in transit amplifying progenitor cells and that differentiation and senescence programs may be linked through DNA-damage responses. This then raises the interesting possibility that replication-mediated DNA-damage may also contribute to stem cell self-

renewal defects, a possibility which appears to hold true for mesenchymal osteogenic progenitors in Bmi1-null mice⁵².

The findings presented herein consolidate a function for PRC1 at the cross road of transcription and replication. Our observations findings provide important connecting points for further research and have important implications for our mechanistic understanding of PRC1 proteins in embryogenesis and oncogenesis.

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Supplemental Materials and methods

Cell culture

ATDC5 cells were cultured at 37 °C, 5% CO₂, 100% humidity in DMEM/ F-12 supplemented with 5% fetal calf serum (FCS), antibiotics (100 units/ml penicillin and 100 µg/ml streptomycin), 200 mM L-glutamine on tissue culture plates (Greiner Bio-One). For differentiation experiments cells were seeded at 6400 cells/cm² and were allowed to attach overnight. Growth medium was changed for differentiation medium, which includes ITS (10 µg/ml insulin, 10 µg/ml transferrin and 3 x10⁻⁸M sodium selenite). Differentiation medium was replaced every two days.

Retroviral infections

Ecotropic retroviral supernatants were produced by transfection of phoenix cells with calcium-phosphate precipitation; 24-48 hours post-transfection, the supernatant was harvested, filtered and used for infection of ATDC5 cells in presence of 4µg/ml polybrene. Cells were incubated for 12 hours and then allowed to recover for 24 hours on fresh medium before selection pressure was applied. Infected cells were selected with puromycin (8 µg/ml) for 72 hours, before experiments were initiated. Short-hairpin (sh)RNA target sequence for shBMI1hm 5'- GTTCAACAAGACCAGACCAC -3', shBMI1h 5'- GAATGGTCCACTTCCATTG-3', shRNF2 5'- GTATCTGGCTGTGAGGTTA-3', shPHC2 5'- GTTCAAGCGTTCCAAGCGC-3'. Short hairpin sequence targeting shGFP was used as reference sh sequence⁵⁷.

Chrystal violet

ATDC5 cells were plated in 12-multiwell plates (Greiner Bio-one). At each time point cells were washed twice with phosphate-buffered saline, and fixed for 10 minutes with 3.7% formaldehyde at room temperature. Next, cells were rinsed 5 times with demiwater. Cells were stained with 0.1% X-tal violet for 30 minutes or overnight, respectively, and washed 5 times with demiwater. X-tal was extracted with 10% acetic acid and absorbance was measured spectrophotometrically at 590 nm (Benchmark, Biorad).

RNA isolation and cDNA synthesis

Cells were suspended in Tri-reagent, and RNA was extracted according to the protocol of the manufacturer (Sigma). Quantity and purity of the RNA were determined by 260/280 nm absorbance measurements. cDNA was synthesized using iScriptTM cDNA synthesis kit (Biorad). Primers used are described in supplemental figure 2.

Western analysis

Cells were lysed in RIPA buffer containing 5 mM Benzamidine, 5 µg/ml Antipain, 5 µg/ml Leupeptin, 5 µg/ml Aprotinin, 1 mM Sodium Vanadate, 10 mM Sodium Fluoride, 10 mM Pyrophosphate, 10 mM β-glycerophosphate, 0.5 mM DTT and 1mM PMSF. Lysates were

subjected to 3 freeze-thaw cycles in liquid nitrogen, and to 3 cycles of sonication. After centrifugation for 10 minutes at 13.2k rpm, protein concentration was determined using a BCA protein assay kit (Pierce). Equal amounts of protein were boiled in Laemmli buffer and loaded on 9-15% polyacrylamide gels. Proteins were transferred to polyvinylidene fluoride (PVDF) membranes. After blocking with 5% non-fat dry milk in PBS containing 0.1% Tween-20, membranes were incubated o/n at 4°C with the following antibodies: anti β -actin Mab (C4, 691001, MP Biomedicals), anti Gapdh, anti Ezh2 Mab (BD43; courtesy D. Pasin Copenhagen, Denmark), anti Bmi1 Mab (F6; Courtesy M. van Lohuizen; Amsterdam, The Netherlands), anti γ H2A.X (Ser139) Poab #2577 (Cell signaling), anti H3K27me3 Poab, #07-449 (Upstate) and anti H3K9me3 #07-442 (Upstate). After extensive washing, membranes were incubated with corresponding horseradish peroxidase conjugated secondary antibodies for 1h at room temperature. Signals were detected using enhanced chemoluminescence.

Immunofluorescence

Briefly, cells were washed and fixed for 10-15 minutes in 100% methanol at -20°C and stored at 4°C in 70% ethanol or used directly for immuno-histochemistry. Cells were permeabilized for 5-10 minutes in 0.2% triton-X in PBS. To prevent epitope loss in combination with acid treatment for BrdU detection, primary antisera were incubated for 1.5 hours at 37°C, 4-5x washed in 0.02% triton-X/PBS and fixed in 2% formaldehyde/PBS for 10 minutes at room temperature. Cells were washed with PBS and incubated for 20 minutes in 2.0N HCl at 37°C, followed by two rinses of 0.1 M sodium tetraborate solution, pH 8.5 for in total 2 minutes. Cells were then incubated with the primary antisera against BrdU, washed at indicated before and incubated with secondary fluorescently labeled antibodies. All Antisera were diluted and incubated in 0.02% triton-X in PBS. Nuclei were counterstained with 4'-6-Diamidino-2-phenylindole (DAPI) and washed in 0.02% triton-X/PBS. The last wash step was in PBS, upon which cells were dehydrated: 1 minute in 70% ethanol, 2x 1 minute in 100% ethanol and air-dried. Cells were mounted in Vectashield (Vector Laboratories, Inc. Burlingame, CA) and analyzed using a NIKON TE200 Eclipse fluorescence microscope and photographed using a NIKON DXM1200 digital camera in combination with NIS Elements 3.0 Imaging software. The following antibodies were used: anti γ H2A.X Mab JBW301 (Upstate), anti BrdU (BD biosciences), anti H3K9me3 #07-442 (Upstate), anti H3K27me3 Poab, #07-449 (Upstate), anti Bmi1 Mab (F6; Courtesy M. van Lohuizen; Amsterdam, The Netherlands), anti H3S10Ph Poab #06-570 (Upstate), anti PcnA PC10 Poab (Chemicon International), anti Phc1 (03041; Courtesy of H. Koseki), anti pRNA polymerase II Mab, ab24759 (Abcam), anti Rad51 Poab sc-8349 (Santa Cruz), anti pKap1 (S824) Poab A300-767A (Bethyl Laboratories), anti RPA70 Poab ab12320 (Abcam) and anti Geminin Poab (FL-209) sc-13015 (Santa Cruz).

Affymetrix expression arrays

A total of 48 Affymetrix NuGO mouse arrays (~24k) were run on RNA from cell cultures sampled over time at 0, 2, 4, 8 hours and 1, 6, 10, 15 days post induction of differentiation (*pid*). For each time point 3 samples were taken from both a wildtype (WT) and a Bmi1 knock-down (KD) culture. After scanning, data preprocessing and analysis were done using R2.7.1 with the use of Bioconductor libraries^{53,54}. First, data were ²log transformed and normalized using gcRMA⁵⁵. Hereafter several statistics were computed for each reporter on the arrays: the average (²logged) expression for each group at each time point, the logratio (²log fold change, logFC) between the two groups at each time point, and the t- and p- value of a Student t-test at each time point. After computing statistics, subsets of reporters fulfilling specific criteria based on these statistics were selected for the several analyses, where generally cut-offs on p-value, logratio, and minimum expression were imposed and controls removed from the data set. Unless otherwise specified, the cut-off limits had to be met at either 1day *pid* or 6 days *pid*. Specific cut-off limits chosen are indicated where applicable in the paper. Overrepresentation of biological processes was determined using PathVisio, using the pathways available from WikiPathways^{56,57}.

Figure S1. Expression analysis of PRC1 complex genes. (A) mRNA expression of PRC1 complex genes during chondrogenesis by microarray analysis. (B) Western blots confirming functionality of short hairpins designed to target PcG mRNAs for RNAi mediated degradation (C) quantification of Bmi1 protein levels shown in figure 1b. (D) qRT rtPCR analysis of collagen 10A1 mRNA expression during differentiation in ATDC5 cells overexpressing Bmi1 and control cells.

Figure S2. PRC1-LOF cells accumulate in S-phase during transit amplification.

(A) ATDC5 cells overexpressing Bmi1 show enhanced hyperproliferation upon induction of chondrogenic differentiation compared to control cells. Proliferation determined by Crystal violet incorporation with subsequent extraction and spectrophotometric analysis. (B) Representative cell cycle profiles of ATDC5 shControl and shBmi1 at t=0 and t=6d *pid*. Values are percentage of S-phase cells compared to total cell population, in addition quantification S-phase positive cells of total experiment. (C) Proliferation of ATDC5 cells expressing shControl, shBmi1 and shPhc2 upon induction of chondrogenic differentiation. Proliferation determined by Crystal violet incorporation with subsequent extraction and spectrophotometric analysis. (D) overview of growthplate. RZ= Resting Zone, PZ= Proliferation Zone, pre-HZ= pre-Hypertrophic Zone, HZ= Hypertrophic Zone, the transition from cartilage to bone is located between HZ and mineralization.

Figure S3. Accumulation of DNA damage in shBmi1 cultures.

(A, B, C) Immunofluorescence staining for γH2A.X in ATDC5 shControl and shBmi1 cultures at varying densities. DAPI used as nuclear staining. (D) Quantification of S-phase positive cells in ATDC5 shControl, shBmi1 and shPhc2 cultures 0 and 6 days *pid*. (E) ACAN mRNA expression measured by qRT rtPCR in ATDC5 shControl, shBmi1 and shPhc2 throughout differentiation.

Figure S4. Bmi1-LOF results in activation of DNA damage responses and correlates to proliferative arrest. (A-D) Immunofluorescence analysis for indicated proteins in ATDC5 shControl and shBmi1 at t=3 *pid*. DAPI is used as nuclear staining.

Gene/acronym	fold	description	Processes involved in
Ifi202	4,9	interferon activated gene 204	tracr//tracr//tracrPol III// transport // // // DDR // Diff
E130016E03Rik	4,1	RIKEN cDNA E130016E03 gene	Dre//DDR
Brip1	3,5	BRCA1 interacting protein 1	Nucmet//Dre//tracrPol III// DDR
Fancb	2,5	Fanconi anemia, complementation group B	Dre//DDR
Rad9	2,4	RAD9 homolog (S. pombe)	CC // CC checkpoint // DDC // Dre//DDR//RadR//apop
Pttg1	2,3	pituitary tumour transforming 1	Dme//Dre//DDR//CC // Cseg//mit//Cseg//biog
Rad51	2,2	RAD51 homolog	homR//Dme//REP//Dre//DDR//mei//meiR
Hspa1b	2,2	heat shock protein 1B	TEL//Dre//fold // antiapop//UPR//hs//
Xrcc2	2,1	X-ray repair complementing defective repair	Dme//Dre//Dre//DDR
Rad51ap1	2,1	RAD51 associated protein 1	homR//Dre//Dre//DDR
Ddb2	2,0	damage specific DNA binding protein 2	Dre//pyrimidine dimer repair //DDR
Exo1	1,9	exonuclease 1	Nucmet//Dre//NER//MMR//Dre//DDR//mei
Blm	1,9	Bloom syndrome homolog (human)	REP//Dre//Dre//
Eme1	1,8	essential meiotic endonuclease 1 homolog 1	Dre//Dre//DDR
Chaf1a	1,8	chromatin assembly factor 1, subunit A (p150)	REP//Dre//tracr//tracr//fold//DDR//CC
Sgk	1,8	serum/glucocorticoid regulated kinase	kin//apop//DDR
Hspa1a	1,8	heat shock protein 1A	TEL//Dre//fold // UPR//hs
Brca2	1,8	breast cancer 2	homR//Dre//chrom//DDR//S-CC//mitC//tracr
Brca2	1,8	breast cancer 2	homR//Dre//chrom//DDR//S-CC//mitC//tracr
Rad51l1	1,8	RAD51like 1	Dme//Dre//Dre//DDR
Cdc2a	1,7	div cycle 2 homolog A	kin//antiapop//CC//mit//G2-C//div
Trex1	1,7	three prime repair exonuclease 1	REP//Dre//MMR//Dre//DDR
Fen1	1,7	flap structure specific endonuclease 1	REP//DNA repair
Msh3	1,6	mutS homolog 3	Dme//Dre//MMR//DDR//somH//somR
Gtf2h1	1,6	general tracr factor II H, polypeptide 1	Dre//tracr//tracr//DDR
Clspn	1,6	clasp homolog	DRC//Dre//DDR//CC
Rad50	1,5	RAD50 homolog	Dme//Dre//DDR//CC//mei
Mapk1	1,5	mitogen activated protein kinase 1	kin//DDR//CC//ST//mor//kin//kin//cytosinemet//diff
Topors	1,4	topoisomerase I binding, arginine/serine rich	Ubd//apop//DDR//me//pro//tracr//trapo
Lig1	1,4	ligase I, DNA, ATP dependent	REP//Dre//Dre//DDR//CC//div
Gadd45a	1,4	growth arrest and DNA damage inducible 45 alpha	regulation of progression through G2C//Pase//DDR//CC//CC arrest
Rfc5	1,4	replication factor C (activator 1) 5	REP//DNA repair
Eef1e1	1,4	eukaryotic translation elongation factor 1 epsilon 1	CC//Dre//tracr//tracr//apop//DDR
Gtf2h2	1,4	general tracr factor II H, polypeptide 2	Dre//tracr//tracr//DDR//tracr
Xab2	1,3	XPA binding protein 2	blast//Dre//TCR//tracr//Rproc
Uvrh	1,3	UV radiation resistance associated gene	Dre
Fanca	1,3	Fanconi anemia, complementation group A	Dre//DDR//malemei//Mgon//Fgon//prol
Bre	1,3	brain and reproductive organ expressed protein	Ubd//apop//DDR//anti-apop
Pold1	1,3	polymerase (DNA directed), delta 1, catalytic subunit	S-CC//Dre//REP//BER//REP
Topbp1	1,3	topoisomerase (DNA) II beta binding protein	Dre//DDR//meiR
Msh5	1,3	mutS homolog 5	Dme//MMR//mei//mei//syn//Fgam
Hmgb2	1,3	high mobility group box 2	REP//REP//Dre//BER//chrom//nuc//tracr//tracrPol II
Parp2	1,3	poly (ADP-ribose) polymerase family, member 2	Dre//BER//ribos
Smc3	1,2	structural maintenance of chromosomes 3	Dme//Dre//DDR//CC//spin//Cseg//SCQ//mit//mei//ST//biog//div
Smc5	1,2	structural maintenance of chromosomes 5	Dme//Dre//Dre//DDR

Table S1. Activation of DNA damage response in Bmi1-LOF cultures.

Expression array analysis of DNA damage response genes presented as log fold change. Associated processes based on Genmapp.

Figure S5. DNA damage and senescence in chondrogenesis. (A+C) Immunofluorescence analysis in ATDC5 shControl and shBmi1 of γ H2A.X and H3K9me3 at $t=3d$ *pid*. DAPI is used as nuclear staining. (B,F) Expression array analysis of GLB1 and JMJD3 in ATDC5 shControl and shBmi1 during differentiation. (D) Senescence-associated β Galactosidase staining on chondrogenic cell lines. (E) Western blot analysis of H3K27me3 and Ezh2 in ATDC5 shControl and shBmi1 during differentiation. β Actin is used as a loading control. (G) Expression array analysis of senescence associated genes in ATDC5 shControl and shBmi1 during differentiation. (H) qRT rtPCR analysis of mRNA expression of chondrogenic markers in ATDC5 expressing shControl, shBmi1, vector or Rasv12 throughout differentiation.

Figure S6. Growth plates of longitudinal bones are positive for SAbgal. (A,B) Representative senescence-associated bGalactosidase staining on longitudinal bones and ribcage/spinal column of wild-type mice. (C) Representative pictures of femur growth plates of Ring1a^{+/+} and Ring1a^{-/-} mice. Brackets indicate hypertrophic zone.

E

Pathway	r	n	total	%	Z
Cholesterol Biosynthesis	10	13	15	76,9	4,66
Cell cycle	32	70	88	45,7	4,62
TGF-beta Receptor Signaling Pathway	44	115	149	38,3	4,03
Androgen Receptor Signaling Pathway	33	84	108	39,3	3,65
Irinotecan Pathway	7	10	13	70,0	3,56
G1 to S cell cycle control	22	51	64	43,1	3,49
Endochondral Ossification	23	56	68	41,1	3,29
TGF Beta Signaling Pathway	18	41	52	43,9	3,24
mRNAprocessing	102	349	552	29,2	3,06
Mm Selenium metabolism/Selenoproteins	18	43	49	41,9	3,00
Apoptosis Modulation by HSP70	9	17	18	52,9	2,97
TNF-alpha/NF-kB Signaling Pathway	47	143	177	32,9	2,94
DNA Replication	15	36	41	41,7	2,71
Heme Biosynthesis	5	8	9	62,5	2,68
One Carbon Metabolism	10	23	41	43,5	2,37
Eukaryotic Transcription Initiation	15	39	41	38,5	2,35
Mitochondrial LC-FattyAcid Beta-Oxidation	6	13	16	46,2	2,01
Apoptosis Mechanisms	24	74	86	32,4	2,00

F

pathways	(r)	(n)	total	%	Z Score
Irinotecan Pathway	4	10	13	40,00%	10,28
Mm Selenium metabolism/Selenoproteins	5	43	49	11,63%	5,67
Endochondral Ossification	5	56	68	8,93%	4,77
Oxidative Stress	2	23	29	8,70%	2,94
Osteoblast	1	7	11	14,29%	2,86
GPCRs, Class B Secretin-like	1	10	13	10,00%	2,28
Osteoclast	1	13	18	7,69%	1,9
TGF-beta Receptor Signaling Pathway	4	115	149	3,48%	1,89

pathways (down)	(r)	(n)	total	%	Z Score
Irinotecan Pathway	4	10	13	40	10,28
Selenium metabolism/Selenoproteins	5	43	49	11,6	5,67
Endochondral Ossification	5	56	68	8,9	4,77
Oxidative Stress	2	23	29	8,7	2,94
Osteoblast	1	7	11	14,3	2,86
GPCRs, Class B Secretin-like	1	10	13	10	2,28

pathways	(r)	(n)	total	%	Z Score
Irinotecan Pathway	5	10	13	50	7,59
Endochondral Ossification	9	56	68	16,1	4,79
Oxidative Stress	5	23	29	21,7	4,47
Cytokines and Inflammatory Response	4	21	25	19,1	3,63
TGF Beta Signaling Pathway	6	41	52	14,6	3,61
Selenium metabolism/Selenoproteins	6	43	49	14	3,46
Osteoblast	2	7	11	28,6	3,4
Prostaglandin synthesis and regulation	4	24	31	16,7	3,27
Adipogenesis	10	108	132	9,3	2,97
Osteoclast	2	13	18	15,4	2,16
Complement activation, classical pathway	2	14	16	14,3	2,03

pathways (up)	(r)	(n)	total	%	Z Score
Adipogenesis	10	108	132	9,3	4,48
Prostaglandin Synthesis and Regulation	4	24	31	16,7	4,38
TGF Beta Signaling Pathway	5	41	52	12,2	3,92
Cytokines and Inflammatory Response	3	21	25	14,3	3,4
Oxidative Stress	3	23	29	13	3,19

Figure S7. Fold change increases during transit amplification

(A) Pie charts of all reporters fulfilling $p < 0.05$, $FC > 1.1$ up or down, expression $> 2^{\log}(100)$ in either group, for each time point in the experiment. (B) The table indicates for each time point: ^athe number of reporters reaching the expression limit of $2^{\log}(100)$ in either group; ^bthe number fulfilling significance ($p < 0.05$), $FC > 1.1$ (respectively 1.2) up or down, and expression above limit; ^cthe percentage of the latter versus the total number of expressed reporters. The figure is a graphical representation of the values in columns ^c. (C) Heatmap of the logratios of all reporters fulfilling $p < 0.05$, $FC > 2$ (left) or $FC < 0.5$ (right), expression $> 2^{\log}(100)$ in either group, at 1 or 6 days *pid*. Euclidean distance and complete linkage hierarchical clustering were used to cluster and reorder the reporters. (D) Profile analysis of the most extremely de-regulated genes identified in expression arrays (up or down). (E) Biological pathways that are overrepresented based on Pathvisio analysis using as criteria: $p < 0.05$ WT vs. Bmi1-KD, $FC > 1.2$ up or down WT vs. Bmi1-KD, and average group expression $2^{\log}(100)$ in either group, at 1 or 6 days *pid*, as compared to all reporters on the array for which the Affymetrix ID (or Unigene ID) could be mapped to a pathway (F) Biological pathways that are overrepresented based on Pathvisio analysis using as criteria: $p < 0.05$ WT vs. Bmi1-KD, $FC > 1.2$ up or down WT vs. Bmi1-KD, and average group expression $2^{\log}(100)$ in either group, at 1 or 6 days *pid*, as compared to all reporters on the array for which the Affymetrix ID (or Unigene ID) could be mapped to a pathway.

Figure S8. PRC1-LOF cultures are more sensitive to replication stress

(A) ATDC5 cells stably expressing shControl, shBmi1, shPhc2, shRnf2, vector or overexpress mBmi1 are exposed to 0 or 50 µg/ml etoposide for 1 hour, washed and allowed to recover for 9 days. Proliferation determined by Crystal violet incorporation with subsequent extraction and spectrophotometric analysis. (B,C) Co-staining for PcnA and BrdU or PcnA and pRNA polymerase II in ATDC5 shControl or shBmi1 at 2 days *pid*.

Chapter 5

Supplemental Table 2

Primers use for qRT rtPCR

Cyclophilin	Forward	CAAATGCTGGACCAAACACAA
	Reverse	TTCACCTTCCCAAAGACCACAT
Bmi1	Forward	TGGAGAAGAATGGCCCACT
	Reverse	GCAAGTTGGCCGAACTCTGT
Collagen 2a1	Forward	CAAGAACCCTGCTCGCACTT
	Reverse	CCGCTCTTCCACTCGGG
Aggrecan	Forward	CATGAGAGAGGCGAATGGAA
	Reverse	TGATCTCGTAGCGATCTTTCTTCT
Collagen 10a1	Forward	CATGCCTGATGGCTTCATAAA
	Reverse	AAGCAGACACGGGCATACCT

Chapter 6

Proliferative Capacity Defines Sensitivity to Valproic Acid During Chondrogenesis.

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Abstract

Valproic acid (VPA) is a drug used to treat epilepsy, schizophrenia and other neurological conditions. VPA administration during pregnancy can have severe adverse effects on the unborn child, including neural tube defects and skeletal malformations. During development stem cells undergo lineage commitment and ultimately adopt terminally differentiated states. Epigenetic regulation, *i.e.* chromatin remodeling, controls gene-environment interactions during development. Although recently VPA was identified as an epigenetic drug, the mechanism(s) of action for VPA remain unclear. VPA has been studied in the context of chondrogenesis, yet published studies report conflicting results. In this study we use the widely used ATDC5 model to study gene-environment interactions in the context of chondrogenesis and to elucidate effects of VPA treatment on chondrogenic differentiation. We find that VPA treatment severely affects chondrogenesis: ATDC5 fail to induce the transcriptional reprogramming required for chondrogenesis. Further, VPA affects differentiation induced hyperproliferation in a TP53 dependent manner. Relevantly, VPA treatment in ATDC5 cells causes replication stress, DNA damage response and results in a senescence-like response. We show that epigenomic remodeling is essential for proper induction of differentiation programs. Our findings support the notion that VPA treatment can be useful to sensitize rapidly proliferating cells to replication stress, providing therapeutic applications for cancer treatment.

Introduction

Valproic acid (VPA) is a pharmacological compound widely used to effectively treat convulsive disorders, migraine, bipolar disorders and schizophrenia. VPA use during pregnancy, however, causes severe developmental abnormalities, both in humans and various laboratory animals: teratogenic effects in mice include neural tube defects, as anencephaly and spina bifida, and skeletal malformations, predominantly along the anteroposterior (AP) axis¹⁻⁶. The outcome of VPA treatment is cell type dependent: rat embryonic neural progenitor cultures (cerebral cortex or hippocampus), show enhanced neurogenesis, and reduced proliferation, accompanied by increased promoter acetylation and expression of neural transcription factors^{7,8}. VPA enhances hepatic and osteogenic differentiation in bone marrow derived and adipose tissue derived stromal stem cell cultures, respectively^{9,10}. Conversely, in various cancer cell types VPA is toxic and leads to senescence and cell death by apoptosis or other mechanisms¹¹⁻¹⁴. VPA toxicity in embryonic stem cells is manifested in reduced cell viability and a potent inhibition of mesodermal and endodermal development¹⁵. Also, at non-cytotoxic concentrations VPA causes abnormal cartilage matrix gene expression in the lumbar spine in chick embryos¹. Although the molecular bases for its effects are poorly understood, experimental studies demonstrated that VPA treatment affects multiple regulatory pathways: Akt activation and consequent glycogen synthase kinase 3 β inhibition have been reported¹⁶, besides stimulatory effect on the GABA and NMDA neurotransmitter receptors¹⁷.

Recently, VPA was shown to directly inhibit histone deacetylases (HDACs)¹⁸. HDACs play an important role in DNA templated processes, such as transcription and replication, through epigenetic remodeling of chromatin. Epigenetic regulation of chromatin structure is widely studied in the context of cancer and development. In pluripotent embryonic stem cells (ESCs) key developmental regulator genes, including Hox genes, display large stretches of repression-associated H3K27me3 and activating H3K4me3 at their promoters. This seemingly conflicting combination of modifications was termed 'bivalency'¹⁹. Differentiation of ESCs is accompanied by remodeling of the epigenome, to facilitate activation of appropriate lineage specific gene expression, and epigenetic silencing of non-lineage specific genes¹⁹. Not surprisingly, many mutant epigenetic regulator genes display developmental defects. Polycomb group (PcG) and Trithorax group (trxG) mutant mice, associated with H3K27me3 and H3K4me3 marks, respectively, are responsible for malformations along the *antero-posterior* (AP) axis. Skeletal phenotypes correlate with deregulation of expression boundaries within HOX gene clusters, well-known PcG and trxG target genes²⁰⁻²². Combined, this demonstrates how proper epigenetic regulation is pivotal to proper cellular differentiation.

Surprisingly little is known about the molecular mechanisms by which VPA affects endochondral ossification (formation of an ossified skeleton from a cartilage scaffold). VPA treatment *in utero* was reported to affect expression of Hox genes and PcG genes in whole mouse embryos^{3,4,23}. The

mature ossified skeleton of mammals arises from a cartilage scaffold. However, direct effects of VPA on chondrogenesis remain poorly understood. The present study therefore aimed to further elucidate effects of VPA in development, with a focus on epigenetic deregulation during chondrogenesis. ATDC5 cells are a widely used model to study chondrogenesis. A remarkable feature of ATDC5 cell differentiation is an early, transient hyperproliferative burst upon induction of differentiation. This phase reflects the transit amplification that mesenchymal progenitor cells undergo during development. We assessed the effects of VPA on ATDC5 chondrogenic differentiation and proliferation. Briefly, we find that VPA specifically impedes proliferation of transit amplifying cells in a Trp53(P53)-dependent manner. In addition, VPA completely abolishes chondrogenic marker gene expression, demonstrating a direct and dramatic effect on chondrogenesis. We here discuss these effects in the context of altered epigenetic control.

Materials & Methods

Cell culture and VPA treatment

ATDC5 cells were cultured at 37 °C, 5% CO₂, 100% humidity in DMEM/ F-12 supplemented with 5% fetal calf serum (FCS), antibiotics (100 units/ml penicillin and 100 µg/ml streptomycin), 200 mM L-glutamine on tissue culture plates (Greiner Bio-One). For differentiation experiments cells were seeded at 6400 cells/cm² and were allowed to attach overnight. Growth medium was changed for differentiation medium, which includes ITS (10 µg/ml insulin, 10 µg/ml transferrin and 3 x10⁻⁸M sodium selenite). Differentiation medium was replaced every two days. VPA (Sigma, P4543) was prepared freshly and supplemented to differentiation medium to pharmacologically relevant end concentrations of 0.3, 1 and 3 mM (FDA).

Retroviral infections

Ecotropic retroviral supernatants were produced by transfection of phoenix cells with calcium-phosphate precipitation; 24-48 hours post-transfection, the supernatant was harvested, filtered and used for infection of ATDC5 cells in presence of 4µg/ml polybrene. Cells were incubated for 12 hours and then allowed to recover for 24 hours on fresh medium before selection pressure was applied. Infected cells were selected with puromycin (8 µg/ml) for 72 hours, before experiments were initiated. Short-hairpin (sh)RNA vector used for RNA interference-mediated knock-down of murine Trp53 was described elsewhere²⁴. Short hairpin sequence targeting shGFP was used as reference sh sequence²⁵.

Chrystal violet and Alcian blue stainings

ATDC5 cells were plated in 12-multiwell plates (Greiner Bio-one). At each time point cells were washed twice with phosphate-buffered saline, and fixed for 10 minutes with 3.7% formaldehyde at room temperature. Next, cells were rinsed 5 times with demiwater. Cells were stained with 0.1% X-

tal violet or 0.1% Alcian blue for 30 minutes or overnight, respectively, and washed 5 times with demiwater. X-tal was extracted with 10% acetic acid and Alcian blue with guanidine/HCL (6M) and absorbance was measured spectrophotometrically at 590 nm or 645 nm, respectively (Benchmark, Biorad).

Senescence-associated β -galactosidase (SA-bGal) assay

Staining was performed according to protocol described by Dimri et al. [26]. Shortly, cells were washed in PBS, fixed for 3-5 min (room temperature) in 2% Formaldehyde /0.2% glutaraldehyde, washed, and incubated at 37°C (ambient CO₂) with fresh senescence associated (3-Gal (SA-,3-Gal) stain solution: 1 mg of 5-bromo-4-chloro-3-indolyl P3-D-galactoside (X-Gal) per ml (stock = 20mg of dimethylformamide per ml)/40 mM citric acid/sodium phosphate, pH 6.0/5 mM potassium ferrocyanide/5 mM potassium ferricyanide/150 mM NaCl/2 mM MgCl₂. SA-bGal activity was measured after 12-16 hours. Staining at pH 4.0 was used as negative control; this detects lysosomal galactosidase activity.

RNA isolation and cDNA synthesis

Cells were suspended in Tri-reagent, and RNA was extracted according to the protocol of the manufacturer (Sigma). Quantity and purity of the RNA were determined by 260/280 nm absorbance measurements. cDNA was synthesized using iScript™ cDNA synthesis kit (Biorad). Primers used are listed in supplemental Table 1.

Western analysis

Cells were lysed in RIPA buffer containing 5 mM Benzamidine, 5 µg/ml Antipain, 5 µg/ml Leupeptin, 5 µg/ml Aprotinin, 1 mM Sodium Vanadate, 10 mM Sodium Fluoride, 10 mM Pyrophosphate, 10 mM β -glycerophosphate, 0.5 mM DTT and 1mM PMSF. Lysates were subjected to 3 freeze-thaw cycles in liquid nitrogen, and to 3 cycles of sonication. After centrifugation for 10 minutes at 13.2 krpm, protein concentration was determined using a BCA protein assay kit (Pierce). Equal amounts of protein were boiled in Laemmli buffer and loaded on 9-15% polyacrylamide gels. Proteins were transferred to polyvinylidene fluoride (PVDF) membranes. After blocking with 5% non-fat dry milk in PBS containing 0.1% Tween-20, membranes were incubated o/n at 4°C with the following antibodies: anti β -actin Mab (C4, 691001, MP Biomedicals), anti Ezh2 Mab (BD43; courtesy D. Pasin Copenhagen, Denmark), anti Bmi1 Mab (F6; Courtesy M. van Lohuizen; Amsterdam, The Netherlands), anti H3K9Ac Poab (#06-599, Upstate Biotechnology) and anti H4K12Ac Poab (ab1761, Abcam). After extensive washing, membranes were incubated with corresponding horseradish peroxidase conjugated secondary antibodies for 1h at room temperature. Signals were detected using enhanced chemoluminescence.

Immunofluorescence

Briefly, cells were washed and fixed for 10-15 minutes in 100% methanol at -20°C and stored at 4°C in 70% ethanol or used directly for immuno-histochemistry. Cells were permeabilized for 5-10 minutes in 0.2% triton-X in PBS. Primary antisera were incubated for 1.5 hours at 37°C, 4-5x washed in 0.02% triton-X/PBS and fixed in 2% formaldehyde/PBS for 10 minutes at room temperature. Cells were washed with PBS and were then incubated with the primary antisera for 1.5 hours at 37°C, 4-5x washed in 0.02% triton-X/PBS, and incubated with secondary fluorescently labeled antibodies. All antisera were diluted and incubated in 0.02% triton-X in PBS. Nuclei were counterstained with 4'-6-Diamidino-2-phenylindole (DAPI) and washed in 0.02% triton-X /PBS. The last wash step was in PBS, upon which cells were dehydrated: 1 minute in 70% ethanol, 2x 1 minute in 100% ethanol and air-dried. Cells were mounted in Vectashield (Vector Laboratories, Inc. Burlingame, CA) and analyzed using a NIKON TE200 Eclipse fluorescence microscope and photographed using a NIKON DXM1200 digital camera in combination with NIS Elements 3.0 Imaging software. The following antibodies were used: anti γ H2A.X Mab JBW301 (Upstate), anti p-Kap1S824 Poab (A300-767A, Bethyl laboratories) and anti H3K9me3 #07-442 (Upstate).

Cell cycle profiles

Cells were fixed in cold methanol overnight at -20°C, washed and resuspended in PBS containing 100 μ g/ml RNase and 20 μ g/ml PI for 2-3 hours at RT in dark environment, immediately prior to FACS analysis. Analysis was performed using a Becton Dickinson FACS machine with CellQuest software.

Results

VPA inhibits chondrogenic differentiation

To examine how VPA affects chondrogenesis ATDC5 cells were differentiated in the presence of neuropharmacologically relevant concentrations of VPA (range: 0.3, 1, 3.0 mM; therapeutic plasma concentrations: 0.3mM – 1mM (FDA)). Starting cultures with low cell density were exposed to a combination of chondrogenic factors (Insulin, Transferrin, Selenite; ITS) and followed for 2 to 3 weeks. Chondrogenic differentiation was assessed by multiple parameters: chondrogenic marker gene expression, progenitor cell proliferation and alterations in morphological appearance. Under the experimental conditions applied in this study, VPA robustly inhibits differentiation even at the lowest concentration, as measured by realtime rtPCR of chondrogenic markers aggrecan (AGC), collagen type II (COL2A1) and collagen type X (COL10A1) after 10 and 15 days of differentiation

(Figure 1A-C). Alcian blue staining for cartilage glycosaminoglycans confirmed the differential effects of VPA on low and high density ATDC5 differentiation (see below; Figure 3B).

Differentiating ATDC5 monolayers undergo a phase of hyperproliferation, and form three-dimensional cartilage nodules [27]. VPA inhibits hyperproliferation during ATDC5 chondrogenesis in a dose dependent manner (Figure 1D) Consistent with altered cell

Figure 1. VPA blocks ATDC5 differentiation and proliferation. (A, B, C) qRT rtPCR analysis of chondrogenic markers of VPA treated ATDC5 cells. (D) VPA inhibits proliferation during ATDC5 differentiation. (E) Cell cycle profiles of VPA treated ATDC5 cells at 2 and 6 days of differentiation. (F) Quantification of figure 1E.

cycle kinetics, DNA profiling showed an accumulation of tetraploid cells upon treatment with VPA: at 3.0 mM VPA ATDC5 show an approximately 10-15% increase in the G2/M peak (Figure 1E,F), mainly at the expense of diploid cells. In parallel with a dramatic decline in cell proliferation, Combined these results demonstrate that VPA blocks chondrogenic transcriptional reprogramming as well as differentiation induced hyperproliferation.

VPA induces senescence in differentiating chondrocytes.

VPA treatment was previously shown to induce cellular senescence¹³, a cellular phenotype which is associated with irreversible replicative arrest²⁸. Senescent cultures often display a large-flat cell morphology accompanied by senescence related marker gene expression profiles. To determine

the adverse effect of VPA on cell cycling, we studied cell morphology. Both ATDC5 and primary human chondrocytes display the typical 'large flat cell' morphology upon exposure to VPA (Figure 2A). Indeed, in good agreement with these morphological changes, staining for senescence-associated- β -galactosidase (SA- β Gal/Glb1) activity, a marker closely associated with replicative senescence, confirms a dose-dependent induction of senescence by VPA (see below; Figure 3C)). Expression profiling of senescence-associated marker genes further corroborated the senescence phenotype: *GLB1* expression is upregulated in response to VPA throughout differentiation compared to control cells (Figure 2B). Chop (Ddit3/Gadd153), a protein involved in the unfolded protein response (UPR) and DNA damage responses, both of which are associated with senescence [29-33], is dramatically upregulated upon treatment with VPA (Figure 2C). Recently, elevated interleukin 6 (IL6) expression was established as a robust marker for senescence-associated secretory phenotype (SASP; ^{34,35}). In keeping with this, we find that expression of IL6 was dose-dependently increased in differentiating ATDC5 cells upon treatment with VPA (Figure 2D). Finally, also the cell cycle inhibitor p19^{arf} was dose-dependently upregulated in VPA stimulated ATDC5 cells (Figure 2E), whereas p16^{ink4a}, expressed from the same locus and associated with senescence, did not respond to VPA treatment (FS, JWV; *unpublished data*). Immunofluorescent analysis of VPA exposed cultures reveals phosphorylated histone H2A.X (γ H2A.X) and phosphorylated Kap1, both markers of early DNA damage responses (Figure 2F,G). Taken together, our findings convincingly show that VPA induces morphological changes, transcriptional reprogramming and DNA damage signaling associated with a senescence-like response during chondrogenesis.

Figure 2. VPA induces senescence in differentiating chondrocytes.

(A) VPA induces a proliferative block and large flat cell phenotype. (B-E) qRT rtPCR analysis of genes associated with senescence in VPA treated chondrocytes. (F,G) Chondrocytes treated with VPA (3mM) accumulate DNA damage, measure by γ H2Ax (F) and p-Kap1 (G).

VPA induced inhibition proliferation, but not differentiation, is dependent on TP53

We next investigated the pathways involved in VPA-induced senescence. In the context of oncogene-induced senescence, activation of a DNA damage responses (DDR) is sufficient to induce senescence, however, inactivation of the DDR, e.g. by ablation of Trp53 or ATM, bypasses proliferative arrest^{32,33}. The cell cycle regulators Trp53 and p19^{ARF} are both causally involved in replicative senescence; P19^{ARF} blocks MDM2-induced proteolytic degradation of Trp53^{36,37}. To determine whether the effects of VPA on chondrogenic differentiation are dependent on Trp53, we stably expressed a short hairpin (sh)RNA which targets Trp53 mRNA for degradation^{24,38}. Remarkably, ablation of Trp53 reproducibly enhances proliferation in control cultures (Figure 3A – top panel; FS, JWV *unpublished data*) and induction of chondrogenesis allows for higher production of glycosaminoglycans in absence of P53 (Figure 3B). Loss of Trp53 function (LOF) rescues proliferation in differentiating ATDC5 cells at low VPA concentration range to a proliferative rate observed in normal chondrocytes (0.3 and 1.0 mM VPA; Figure 3A). VPA was reported to induce abnormal nuclear morphology in cancer cells³⁹. We find that VPA also dramatically changes nuclear morphology in chondrogenic cultures at high concentration: nuclei acquire extremely irregular multi-lobed shapes irrespective of Trp53 (Figure 3C; lower panels). We recently discovered that SAbGal is induced as part of normal chondrogenesis (FS, JWV *in preparation*); as such the effect of VPA on SAbGal induction can only be studied under conditions in which VPA induces an acute proliferative arrest (preceding hyperproliferation). 3 mM VPA activates the typical bGal staining observed in senescent cells²⁶. Remarkably, when stained for SAbGal, we find that Trp53-LOF prevents SAbGal activation at the higher VPA concentrations (1.0 and 3.0 mM; Figure 3D), suggesting that transcriptional activation of or signaling to SAbGal is dependent on Trp53.

Surprisingly, Trp53-LOF does not rescue the defective differentiation of VPA treated ATDC5 cells, as measured by Alcian Blue staining for cartilage matrix glycosaminoglycans (Figure 3B) and quantitative RT/rtPCR of chondrogenic markers (Figure 3E): expression of key chondrogenic regulator genes *SOX9* and *RUNX2* is differentially affected by Trp53 or p19^{ARF}-LOF: *RUNX2* mRNA is clearly suppressed at 7-10 days *pid*, whereas *SOX9* expression appears largely unaffected. Conversely, although Trp53 or p19^{ARF}-LOF leads to an exaggerated *SOX9* and *RUNX2* induction at 10 days *pid* (Figure 3E), which correlates with the increased alcian blue staining at this timepoint (Figure 3B). Overall, however, Trp53 or p19^{ARF}-LOF do not rescue terminal chondrogenic capacity as judged by *ACAN*, *COL2A* and *COL10A1* expression and glycosaminoglycan staining (Figure 3F,B). We conclude from this combined data that Trp53-dependent proliferative control is an important component of chondrogenic differentiation, but that chondrogenic transcriptome reprofiling is not controlled by Trp53.

VPA affects epigenetic modifiers and histone modifications

To study possible effects of VPA on epigenetic regulation during chondrogenesis we examined expression on epigenetic regulators belonging to the Polycomb Group proteins in VPA exposed cultures. A recent study implicated reduced Polycomb Group gene expression in abnormal skeletogenesis of VPA-exposed fetuses *in utero*²³. We therefore investigated the effect of VPA on expression of a number of relevant Polycomb genes. Polycomb repressive protein complexes (PRCs) harbor enzymes with intrinsic epigenetic activities, among which histone H3 lysine 27 methyl transferase activity

Figure 3. VPA induced inhibition proliferation, but not differentiation, is dependent on TP53.

(A) Expression of shTP53 rescues part of VPA induced proliferation block. (B) Quantification of alcian blue staining from ATDC5 cells stably expressing shControl and shTP53, in VPA treated (3mM) and non-treated differentiating chondrocytes. (C) Nuclear morphology (DAPI staining) of ATDC5 cells stably expressing shControl and shTP53 treated with VPA (0, 0.3, 1 and 3mM) at day 6 of differentiation. (D) SAbGAL staining on shControl and shTP53 expressing ATDC5 cells treated with VPA (0, 0.3, 1 and 3mM) at day 6 of differentiation. (E,F) qRT rtPCR analysis of chondrogenic markers of VPA treated (0, 0.3 and 1mM) ATDC5 cells expressing shControl, shTP53 or shP19^{ARF}.

(EZH2), histone H2A lysine 119 ubiquityl E3 ligase activity (RNF2, BMI1, RING1). Expression analysis of these factors revealed apparent regulation of PRC gene expression through out chondrogenesis (Figure 4A, white bars), however, transcriptional deregulation in the context of VPA exposure failed to show significant reproducible changes in expression or consensus trends among PRC genes (Figure 4A, shaded bars). Of PRC genes examined, VPA only reduces *BMI1* expression at 6 days *pid*, both at the mRNA and protein level (Figure 4A,B).

Figure 4. VPA affects epigenetic modifiers and histone modifications.

(A) qRT rtPCR analysis of epigenetic modifiers in ATDC5 treated with VPA (0, 0.3, 1 and 3mM) at T=0, 2h, 3d, and 6d. (B) ATDC5 cells were differentiated for 0, 1, 3, 5 and 7 days, treated with VPA (0, 0.3 and 1mM) and analyzed for protein expression of epigenetic modifiers. (C) ATDC5 cells were differentiated for 0, 1, 3, 5 and 7 days, treated with VPA (0, 0.3 and 1mM) and analyzed for expression of histone modifications. (D) qRT rtPCR analysis of Egr1 in ATDC5 with VPA (0, 0.3, 1 and 3mM) at T=0, 2h, 3d, and 6d. (E) VPA dose-dependent increase in H3K9me3 levels in ATDC5 and DAPI nuclear staining

To determine global changes in epigenetic marking as a result of VPA exposure, we analyzed covalent histone modifications throughout chondrogenesis in control cultures (t=0) and compared these to cultures exposed to VPA. Covalent histone modifications were analyzed using specific antisera against acetylated lysine 9 and 14 on histone 3 (H3K9/14ac) or H4K12ac. We find a dose dependent hyperacetylation of histone H3 and 4 in response to VPA (Figure 4C). This global hyperacetylation correlates well with substantially increased early chondrogenic expression of the immediate-early response gene *EGR1*, which was reported to recruit histone acetyl transferases (HAT; Figure 4D). Finally, H3K9 trimethylation, which is associated with cellular senescence (Lowe), is induced in a dose dependent manner by VPA (Figure 4E). Relevantly, nuclei with altered morphology display very high H3K9me3 levels (Figure 4E inset). Combined these data substantiate the importance of epigenetic modulation early in chondrogenesis and confirm a prominent role for VPA during the early stages of chondrogenic differentiation.

Discussion

The widely used anticonvulsant and mood-stabilizing drug VPA is known to cause brain damage and birth defects in the fetus. As the molecular mechanisms underlying VPA-induced AP-axis abnormalities remained incompletely understood, we investigated VPA in the context of chondrogenic differentiation. We report here that VPA treatment strongly inhibits chondrogenic differentiation and blocks progenitor proliferation during the hyper-proliferative phase in chondrogenic differentiation. Furthermore, we show that VPA induces a stress response that activates TRP53-dependent proliferative block during differentiation induced hyperreplication.

Chondrogenic differentiation is a multi-phasic process, which is controlled by a variety of transcription factors, growth and differentiation factors. During the earliest phase of chondrogenic differentiation the cells proliferate rapidly, which is critical for formation and extension of the cartilage scaffolds that ultimate will form bony elements of the skeleton. The Sox-family of transcription factors are key regulators of these early phases of differentiation. Eventually, the differentiating chondrocytes withdraw from the cell cycle and become hypertrophic. Toward terminal differentiation chondrocytes excrete a variety of extracellular matrix proteins (*i.a.* Col2A1; Col10A1; Acan) that will constitute the cartilage matrix. Hypertrophic chondrocytes excrete factors which initiate vascularization and attract osteoblasts. Eventually hypertrophic chondrocytes undergo apoptosis, are replaced by invading osteoblasts, which signals ossification of the cartilage matrix⁴⁰.

Previous reports established axial skeletal transformations in mice exposed to VPA *in utero*^{4,23}. Also in lumbar spines from chick embryos cultured *ex vivo* VPA was found to affect mRNA

expression of collagen IX and Aggrecan, and VPA induced cytotoxicity in mesenchymal cells isolated from the chick spine in a dose-dependent manner¹. Our studies which show a dramatic effect of VPA on chondrogenesis in ATDC5 are in good agreement with these findings. A recent study reported no effects of VPA on ATDC5 chondrogenic differentiation when differentiation was initiated in a high density culture⁴¹. These differences may be explained by differences in experimental procedure: protocols applied in the current study²⁷, induce differentiation in ATDC5 cultures at low density, whereas the reported protocol involved pre-plating of cells and proliferation to confluency, and subsequent induction of ITS-mediated differentiation. The original ATDC5 differentiation protocols revealed a hyperproliferative burst and the subsequent formation of multilayered cartilage nodules²⁷, thus allowing detailed analysis of both proliferation dynamics as well as transcriptome reprogramming. Preliminary comparative analysis of these two differentiation protocols shows that initiation of differentiation at high confluency does not lead to induction of hyperproliferation and formation of cartilage nodules (FS,TW,JWV *unpublished data*), most likely since cells have already ceased cycling at the start of differentiation. Together with our finding reported here, that chondrogenic hyperproliferation is controlled by Trp53, this further supports the notion that hyperproliferation is an essential element during chondrogenesis. Remarkably, Trp53-LOF does not rescue the ability of VPA-exposed cells to differentiate. This points to a distinctive role for Trp53 in cell proliferation, rather than differentiation. Indeed, similar observations were made in zebrafish studies recently⁴². These collective observations underscore the notion that transcriptional reprogramming and proliferation are integrated, yet distinctively regulated processes during chondrogenesis.

In addition to apoptosis, senescence is increasingly recognized as a mechanism to protect cells from oncogenic transformation⁴³. Ectopic expression of oncogenes, such as RAS and BRAF in normal diploid fibroblasts evokes oncogene induced senescence (OIS)^{32,44}. Upon oncogenic activation these cells first undergo a short phase of hyperproliferation, after which replication is markedly reduced and senescence sets in. The origin of replicative senescence is viewed as an inability of cells to keep up with hyperproliferation, which results in DNA damage, stalled replication forks and, importantly, a replication stress-induced DNA damage response (DDR) which triggers DDR dependent OIS; indeed, ablation of DDR (ATM, ATR, Trp53) bypasses aspects of OIS²⁹⁻³³. Analogous to oncogenic activation of fibroblasts, ATDC5 differentiation includes a phase of rapid DNA replication. In keeping with activation of a senescence-like response by VPA, we find a number of senescence markers upregulated in VPA exposed cultures: the tumor suppressor p19^{ARF} is progressively upregulated in VPA exposed chondrogenic cultures. Consistent with this, ATDC5 cells respond to VPA induced stress by undergoing Trp53-dependent proliferative arrest and senescence. P19ARF is known to stabilize Trp53 and early senescent-associated responses are known to involve both factors. In addition, elevation of Chop, also known as DNA damage inducible transcript-3 (Ddit3)/Gadd153, supports a role for DDR in VPA-induced senescence. Our findings are consistent with recent reports that VPA induces a senescent-like phenotype in

medulloblastomas¹¹. In addition, several reports show that VPA induces a proliferative arrest in cancer cells of haematologic origin^{12,45}, which may be associated with activation of autophagocytosis, a phenomenon known to be related to ERS and UPR^{30,44}. The molecular basis of the VPA-induced DDR is not fully clear. VPA was shown to induce a variety of stresses, e.g. ER stress and production of reactive oxygen species (ROS)^{46,47}. However, the mechanisms by which VPA treatment leads to ER stress or ROS production remain elusive. Consistent with the herein reported compromising effect of VPA on the ability of ATDC5 cells to hyper-replicate DNA, others have shown an enhanced sensitivity to the topoisomerase poisons⁴⁸. Finally, we show that H3K9me3 is strongly induced upon VPA exposure, providing an additional link to senescence. Senescent tumor cells show accumulation of H3K9me3⁴⁹. Although the exact role of H3K9me3 in OIS is still not clear⁵⁰, a global increase of H3K9me3 may be part of a cellular stress response (FS, PP, JWV unpublished data).

Despite lack of experimental evidence for a generally reduced Polycomb function, the effect of VPA on chondrogenesis unlikely affects Polycomb gene expression directly, it is possible that abnormal associated epigenetic activity, like blocked HDAC function, contributes to the skeletal phenotype^{1,4,51}. The reduction of the PRC1 protein *BMI1* upon VPA exposure is interesting, in that it is consistent with our recent observation that Bmi1 is a prime regulator of chondrogenesis (FS, JWV; *in preparation*). In addition to possible direct effects on chondrogenesis, Bmi1-LOF was reported to induce senescence^{52,53}; as such the VPA-induced response could be in part explained by defective Bmi1 function.

We here report a concentration dependent hyperacetylation of histones H3 and H4 during the early phases of chondrogenesis in VPA-exposed cells. These observations are consistent with published reports on the effect of VPA on epigenome-wide histone acetylation in various cells¹⁸. It has been speculated that the HDAC inhibiting activity of VPA promotes hyperacetylation leaving chromatin in an open configuration and more susceptible to DNA damage during replication⁴⁸. Interestingly, VPA induces a strongly amplified expression of *EGR1*, an immediate early gene we recently identified as a crucial factor for early epigenomic reprogramming in chondrogenesis (FS, JWV; *in preparation*). Egr1 recruits CPB/p300 HAT activity to promoters. It is conceivable that this exaggerated *EGR1* activation is instrumental in the observed abnormal epigenomic responses. Relevantly, hyperacetylation has been connected to polyploidy, a phenomenon which is strongly associated with senescence^{8,11,54}; Egr1, depending on the cellular context, stimulates or reduces proliferation. The data presented here suggest a molecular connection between Egr1 and VPA in the context of early epigenomic remodeling and between VPA and Bmi1 in the regulation of hyperproliferation during chondrogenesis. The dose dependent effects of VPA on chondrogenesis we report are consistent with epigenetic (de)regulation, as epigenetic modulation most often produces hypomorphic (e.g. variegation or gene-dosage effects) rather than all-or-none effects. In light of findings reported by us and others, it is likely that chondrogenesis (and consequently

skeletogenesis) are specifically sensitive to VPA, because of characteristic biological features associated with endochondral ossification, among which rapid proliferation and hypertrophy.

In conclusion, VPA causes an enhanced susceptibility to replication stress and induces a Trp53-dependent replicative block and senescence-like response in hyperproliferating chondrocytes. Our findings have potentially important repercussion for the clinical use of VPA: the success of combination therapy of e.g. both solid and hematological cancers with VPA and topoisomerase inhibitors^{48,55} may directly relate to enhanced sensitization of rapidly dividing cancer cells for replication stress, in analogy to the effects on chondrogenic progenitors we report here. These combined observations should provide important leads for detailed analysis of the molecular basis of the role of VPA on replication and provide relevant possibilities for therapeutic applications in cancer.

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Supplemental Table 1 Primers used for qRT rtPCR

Cyclophilin	Forward	caaatgctggaccaaacacaa
	Reverse	ttcaccttccaaagaccacat
Collagen 2a1	Forward	caagaaccctgctcgactt
	Reverse	ccgctcttccactcggg
AggreCAN	Forward	catgagagaggcgaatggaa
	Reverse	tgatctgtagcgatcttttct
Collagen 10a1	Forward	catgcctgatggctcataaa
	Reverse	aagcagacacgggcatacct
Glb1	Forward	gagaaagtcaagacagtggtg
	Reverse	ccattgggacacaggataccc
Il-6	Forward	gctacaaactggatataatcaggaaa
	Reverse	ctgttatctttaagttgttctcatgtactc
Chop	Forward	gggccaacagaggtcacac
	Reverse	cttcatgcttgcttcca
P19ARF	Forward	aagacggccttgacaggtcat
	Reverse	ctagtaccggaggtcatcttga
Sox9	Forward	agtaccgcacctgcacaac
	Reverse	tactgtagtccgggtggtcttc
Runx2	Forward	gacgaggcaagagtttcacc
	Reverse	ggaccgtccactgtcactt
Ezh2	Forward	gaaaaaagatgagacgtccagctc
	Reverse	gaggttcaatattggctcatcttat
Bmi1	Forward	tggagaagaatggcccact
	Reverse	gcaagttggccgaactctgt
Rnf2	Forward	tcaggcccatccaactct
	Reverse	ggcattgcctgaagtcttatgt
Ring1	Forward	gccacagtggatcatctctcc
	Reverse	tgctgctgcctcctctcc
Egr1	Forward	agccgagcgaacaaccctat
	Reverse	tgtcagaaaaggactctgtgtca

Chapter 7

General Discussion

PcG proteins are epigenetic regulators, first identified in *Drosophila melanogaster* as regulators of segmental identity. PcG mutant *Drosophila* and mice show homeotic transformations along the antero-posterior axis, in mice resulting in vertebral transformations¹⁻⁴. The skeletal phenotype of PcG mutant mice has been ascribed to deregulation of *HOX* genes, classical PcG target genes, but the precise mechanism behind the development of segment identity shifts is currently unknown. Chondrogenesis is a multistep process that forms the cartilage matrix that will eventually ossify to form skeletal elements¹. We speculated that PcG proteins play a critical role in mediating the epigenomic responses to a changing microenvironment (here inducing chondrogenic differentiation), and thus in cell fate decisions. In this thesis various techniques were used to study chondrogenic differentiation and in particular the role of PcG proteins herein.

We find that a hypoxic response during chondrogenesis is an important part of cellular responses to changing environmental conditions during chondrogenic differentiation (chapter 3). In addition, we find that interference with epigenomic remodelling during chondrogenesis severely affects chondrogenic capacity. This aspect was studied by exposure to Valproic acid (VPA), a reported HDAC inhibitor and by functional loss of important epigenetic regulators of the Polycomb Group (PcG) of proteins. Finally we find that induction of the immediate early response gene *EGR1* is required for epigenomic remodelling during the early phases of differentiation.

Gene-environment interactions in chondrogenic differentiation

In a novel *in vivo* model we show that placing activated mesenchymal stem cells, derived from a periosteal callus (PC), into an induced lesion, repairs the osteochondral defect (Chapter 3). Without supplemental addition of growth factors, relevant pathways are activated and this model recapitulates the different stages of enchondral ossification. Lack of vascularization makes cartilage a very hypoxic tissue environment². In line with this notion we observed increased mRNA expression of HIF-1 α and its transcriptional targets VEGF and GAPDH (Chapter 3). Under normoxic conditions HIF-1 α is hydroxylated by prolyl hydroxylases and consequently ubiquitylated by the von Hippel Lindau gene product VHL, and targeted for proteasomal degradation³. Under hypoxic conditions HIF-1 α cannot be prolyl hydroxylated, resulting in stabilization of HIF-1 α . HIF-1 α helps cells adapt to a hypoxic environment, by activating downstream targets, involved in e.g. cellular survival, anaerobic metabolism and vascularization. We observed stabilization of the HIF-1 α at the protein level in non-hypertrophic chondrocytes. The absence of HIF-1 α protein in hypertrophic chondrocytes is in good agreement with the onset of vascularization, and consequent oxygenation in the hypertrophic region of the growth plate.

In this study, we observed a down regulation of BMP-2, -4, and -7, while increased levels were found at the protein level, throughout callus formation and osteochondral differentiation (Chapter 3). Studies on BMP proteins in callus formation and osteochondral differentiation are not in agreement; while Yaoita et al. reports that levels BMPs remain unaffected in response to inducing a fracture, Kloen et al. reports a positive staining for BMPs in human callus. Others show an up-

regulation of BMP-2 24 hours after surgical release of the periosteum ⁴. The results in the study presented here, show regulation of BMPs at both the transcriptional as well as post-transcriptional level. Recently, select mRNAs were found to be preferentially translated under hypoxic conditions through increased association of mRNAs with polysomes ⁵. Whether BMPs are subject to preferential translation under hypoxia remains to be determined. Combined, these results strongly suggest that the microenvironment, in which the PC is placed, results in activation of pathways relevant to chondrogenic differentiation and to adaptation to the hypoxic surroundings.

Dual role for Egr1 during chondrogenic differentiation

Murine ATDC5 mesenchymal stem cells activated to undergo chondrogenesis reiterate the well-described multi-stage differentiation program, and are widely used to study chondrogenic differentiation. We report for the first time that in response to a changing the microenvironment (i.e. Growth factors in differentiation medium) rapidly and transiently induces *EGR1* and other immediate early genes (IEGs). Knockdown of *EGR1* in ATDC5 cells severely impedes two different characteristics of ATDC5 chondrogenesis.

Firstly, the ability of Egr1-LOF (loss-of-function) cells to undergo chondrogenic differentiation is severely affected. In line with this, promoters of *RUNX2* and *SOX9*, transcription factors critical for different aspects of chondrogenesis were identified as direct targets of Egr1 (Chapter 4); in the absence of Egr1, activation of these targets is blunted upon induction of differentiation. Egr1 was shown to recruit HAT activity ⁶, which is in good agreement with early abnormal global histone acetylation, observed in Egr1-LOF cells. Currently, we do not know whether Egr1 directly activates transcription of these targets, or whether recruitment of HAT activity to relevant promoters creates an epigenomic state permissive of transcriptional activation.

Secondly, ATDC5 cells induced to differentiate undergo a transient phase of rapid progenitor proliferation; Egr1-LOF cells fail to undergo this differentiation induced expansion phase and undergo premature senescence, specifically during the hyperproliferative phase of chondrogenic differentiation (Chapter 5). Senescence is a protective mechanism to prevent oncogenic transformation of cells that accumulate excessive damage. Indeed, loss of Egr1 function results in accumulation of DNA damage and expression of numerous senescence-associated marker genes (Chapter 4). Intriguingly, this proliferative block resembles features of oncogene-induced senescence ⁹⁻¹¹. Expression of oncogenes results in hyperproliferation, and thus hyperreplication, to such an extent that the cells cannot maintain proper regulation of replication ⁷⁻⁹. Consequently, replication forks stall, and DNA damage accumulates. Next, activated DNA damage responses (DDR) induce a cell cycle arrest in S-phase and irreversible senescence, which are dependent on the DDR ⁷.

Our findings position Egr1 at the onset of a proliferative burst. Hence Egr1 fulfils a regulatory role in lineage-specific transcriptional re-programming, as well as in transit progenitor expansion.

Loss of Egr1-function affects epigenomic remodeling

Apart from lack of global acetylation, examination of other histone modifications revealed a significantly increased trimethylation of H3K4, H3K9 and H3K27, both in control and Egr1-LOF cultures. In control cells trimethyl levels increase during the phase of transit amplification. In addition, an exaggerated increase in expression of these modifications is observed in Egr1-LOF cultures. These epigenetic trimethylation marks are seemingly in conflict with each other, as for instance H3K9 and H3K27me3 are associated with transcriptionally repressed/silent DNA, whereas H3K4me3 is often found at loci which are actively expressed. The observed increase in global trimethylation correlates with a period of enhanced cell stress and suggest that besides in transcriptional regulation, these modifications are implicated in other, as of yet unknown regulatory processes. It has been reported that the heterochromatin markers HP1 and H3K9me3 both accumulate in distinct nuclear foci during or in response to cellular senescence ¹⁰. These senescence-associated heterochromatin foci (SAHF) appear during replication stress induced senescence. We find that expression of KAP1 precedes the accumulation of H3K9me3 in Egr1-LOF cells (Chapter 4). Kap1, a factor known to directly bind HP1, was reported to be required to help resolve DNA damage specifically in heterochromatin ¹¹. Together, H3K9me3 and Kap1 may be involved in protection against replication induced DNA damage. Also increased levels of H3K4me3 and H3K27me3 have been associated with cellular stress responses, i.e. hypoxia or induction of replication stress (Johnson et al. +FS, PP, JWV; unpublished data). H3K4me3 was reported to be essential for Rag2-mediated V(D)J recombination in B and T cells, and is linked to DDR-induced cellular responses via ING family proteins. In line with this notion, we also find that VPA, a reported HDAC inhibitor, severely affects chondrogenic differentiation. Like loss of Egr1 and PcG function (chapter 5 + see below) VPA treatment leaves the ATDC5 cells unable to hyperproliferate and activates senescence response. The observed cellular stress responses again correlate with globally enhanced histone trimethylation. Interestingly, VPA treatment affects proliferation, but not on differentiation, in a TP53-dependent manner (Chapter 6). These findings are in agreement with similar observations in zebrafish ¹². Combined these observations suggest that several important processes, including a proliferative response and transcriptomic reprogramming, are part of a coordinated response during chondrogenesis. Our findings support the importance of epigenomic remodelling for chondrogenesis. In addition, they suggest that global trimethylation may be part of a more generalized stress response.

Egr1 initiates early epigenomic reprogramming via activation of PcG proteins

In silico analysis of Egr1 binding sites predicted PcG proteins as potential target genes. Interestingly, we observed that Egr1-LOF affects expression of Ezh2 and Bmi1, and in line with these findings we were able to immunoprecipitate PcG promoter sequences with specific anti-Egr1 antibodies (Chapter 4). Both Ezh2 and Bmi1 have been associated with cell-cycle regulation, as Ezh2 is a transcriptional target of E2F ¹³, and Bmi1 transcriptionally regulates the CDKN2/INK4A

locus, which encodes the cell-cycle regulators P16^{ARF} and P14^{INK4a} ¹⁴. This suggests that Egr1 is involved in transcriptional activation of PcG genes and that Egr1 thereby recruits epigenetic modulators essential for chondrogenic differentiation. Furthermore, a comparison between published PcG targets and predicted Egr1 consensus binding sites revealed potential common targets. Relevantly, two hours post induction of differentiation (*pid*) we observe a strong correlation between H3K27me3 marks at loci and the absence of Egr1 binding, and inversely, between the presence of H3K4me3 marks and local enrichment of Egr1, suggesting that PRCs regulate accessibility of Egr1 to promoters. Combined, this data reveal a number of potential mechanistic interactions between Egr1 and PRCs.

The PRC1 complex regulates transit amplification and transcriptional reprogramming of chondrogenic progenitors

Analogously to Egr1-LOF cells, loss of PRC1 function results in failure to properly differentiate. In addition we observe a significant intra-S-phase arrest and induction of senescence. In agreement with the intra-S-phase arrest, BrdU incorporation revealed an inability to properly replicate DNA in Bmi1-kd cells. Under differentiation conditions, PRC1-LOF cells accumulate massive amounts of DNA damage, as measured by phosphorylated H2A.X, Chk2 and Kap1 (Chapter 5). Again, as for loss of Egr1 function, these results show strong similarity to OIS, suggesting that hyperproliferation induced replication stress is the underlying cause of the phenotype observed in PRC-LOF.

All chondrogenic cell lines available showed strong staining for senescence-associated bGalactosidase (SAbGAL). Furthermore, intercalated discs and in growth plates of elongating bones of WT mice show presence of SAbGAL. In good agreement with in vitro findings, RING1A-KO mice show an increased hypertrophic zone (HZ) (Chapter 5). Importantly, Runx, an oncogene, reported to induce senescence, is implicated in hypertrophic differentiation. Together, our findings suggest that endochondral ossification may naturally terminate in a senescence-like phenotype. To the best of our knowledge, this is the first report describing an important role for hyperproliferation checkpoints in a naturally occurring differentiation process during development. Thusfar these cellular stress responses (DDR, intra-S arrest and senescence) have mainly been associated with oncogene-induced hyperproliferation and consequential checkpoint activation and cell cycle exit.

We performed extensive microarray analysis on Bmi1-kd and control cells, and we found that transcriptional re-profiling in control cells intensifies during transit amplification. Transcriptomic differences between Bmi1-kd and control cells increase dramatically at this time. Unexpectedly, we observed that equal amounts of genes are up- or down-regulated at 6 days *pid* in Bmi1-kd cells compared to control cells (Chapter 5). Expression profiling of genes most extremely deregulated revealed a number of different response clusters, suggesting global deregulation of transcriptional responses rather than deregulation of specific relevant processes. Combined these findings illustrate that at the time of proliferative amplification, the transcriptome is heavily reorganized to

facilitate chondrogenic differentiation, and that PRC1 is critical for regulating these transcriptional changes.

Cells undergoing differentiation require coordinated regulation of DNA-templated processes, among which transcription and replication. Using Immunofluorescence, we studied transcription, replication and the presence of DNA damage in respect to each other. Intriguingly, transcription and replication activity appear to be separated in space and time inside the nucleus, suggesting that the genome is partitioned into regions that are either actively transcribing or actively replicating DNA under normal conditions (Chapter 5). This unexpected finding is in good agreement with an earlier report on dynamic segregation of mRNA and DNA production throughout S-phase¹⁵. Remarkably, this spatial segregation of transcription and replication is lost in PRC1-kd cultures, suggesting that concerted regulation of the two DNA-templated processes is regulated by PRC1. Moreover, in PRC1-LOF cells, DNA damage occurs at sites of *de novo* DNA synthesis. The majority of DNA damage in replicating cells, even under normal conditions, probably comes from highly frequent collisions between replication forks and transcription machineries, as both processes move along the same template¹⁶⁻¹⁸. Our findings suggest that damage in PRC1-LOF cells accumulates as a result of failure to tune replication to transcription and *vice versa*.

Bmi1-deficient cells are positive for geminin expression and control cells are not (Chapter 5). This inverse correlation between Gmnn and Bmi1/Rnf2 chromatin association during mid-S is in perfect agreement with the recently identified ubiquitin-mediated induction of Gmnn degradation by Bmi1/Rnf2¹⁹. Chromatin dissociation of Bmi1/Rnf2 is likely controlled by post-translational modification (PTM). In line with this idea, we recently observed a positive correlation between PRC-phosphorylation, PRC1/chromatin dissociation and Gmnn stabilization under various conditions of cell stress (JWV, YT unpublished observations). Stress signaling induces chromatin dissociation of PRC1 members (Bmi1, Ring and CBX proteins;²²). In contrast, Phc1/Rae28²², through which Gmnn is associated with chromatin, undergoes a nuclear redistribution under these conditions. Taken together, these observations point to Gmnn as a likely candidate for coordinated control of transcription and replication in the context of PRC1 function.

Based on these findings, we propose a model in which the PRC/geminin interactions maintain a dynamic switch to spatially and temporally segregate transcription and replication: H3K27me3-mediated recruitment of the core complex CBX/Rnf2/Bmi1 installs repressive H2AK119ub1 marks, which interfere with transcription initiation, possibly through recruitment of additional PRC (associated) proteins²⁰. Local presence of Rnf2/Bmi1 E3 ligase activity would target Gmnn for degradation, relieving a potential replication block. Conversely, loss of Bmi1/Rnf2 stabilizes GMNN, hence blocking replication, yet permitting transcription (see figure 1, chapter 2).

Conclusions and future perspectives

The research in this thesis provides novel insights to mechanisms mediating gene-environment interactions. Changing the environment to induce chondrogenic differentiation rapidly induces a wide array of responses (Chapters 3, 4 and 5). Egr1, while expressed only shortly, activates chondrogenic programs and initiates epigenomic remodeling. Initiating epigenomic remodeling by Egr1 is critical for subsequent transit amplification of chondrogenic progenitors, at least in part via regulation of PRCs (Chapter 4). The importance of epigenomic remodeling during transit amplification is stressed by the findings that the histone deacetylase inhibitor valproic acid results in replicative senescence and a failure to differentiate (Chapter 6). Detailed analysis of PRC function during chondrogenesis revealed novel insights in coordinated regulation of transcription and replication and proposes a role for PRCs therein (Chapter 5). Loss of PRC function results in replication stress, in accumulation of DNA damage and induction of replicative senescence (Chapter 5). Although our studies have increased our fundamental understanding of gene-environment interactions and uncovered a novel role for PcG proteins in coordinating transcription and replication, many questions remain. May Egr1 binding to DNA define genomic regions with active transcription or replication? Is Egr1 binding restricted to promoter regions? How does Egr1 binding to DNA relate to specific histone modifications, and to PRC occupation? Which genes are regulated by Egr1 or PRCs? Does PRC function in replication timing, and is Egr1 involved? Is enrichment of specific histone modifications and PRC occupation dynamic during S-phase, when transcription and replication are co-ordinately regulated? Are potential targets dynamically regulated in replication timing the genes that accumulate DNA damage in PRC- or Egr1-LOF? How, exactly does geminin contribute to Polycomb biology? Genome wide chromatin immunoprecipitation (ChIP-seq) of Egr1, specific histone modifications and PcG proteins during different stages of differentiation, will be important to map common targets. In combination with microarrays of Egr1-LOF versus control cells and the available Bmi1-LOF microarray data, these data sets will shed light on the importance of regulating these targets, in the context of transcription. Replication timing involves coordinated regulation of transcription and replication (discussed in Chapter 2). This process provides a relevant starting point to study the coordinate regulation of transcription and replication by PRCs. Genome wide ChIPs for active RNA polymerase II, BrdU, geminin and specific histone modifications in different stages of S-phase, as a function of PRC expression will help studying the proposed model (Chapters 2 and 7). Our findings suggest a novel aspect of Polycomb biology during skeletogenesis, and to the importance of proliferation and tissue expansion in positioning along the A-P axis. Further, premature termination of differentiation may account for the phenotype observed in PcG null-mutants.

Overall, our observations provide important connecting points for further research, and contribute to mechanistic understanding concerning the role of PcG proteins in embryogenesis and oncogenesis. Finding answers to the above questions will be of significant importance to enhance our as of yet incomplete understanding of epigenomic regulation in development and disease.

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Summary

Summary

All genetic information that a cell needs to function is stored in its DNA. Genes are transcribed into mRNA, which is subsequently translated into proteins. When a cell prepares for cell division, the nuclear DNA is duplicated and divided between two daughter cells and all genetic information is maintained. During development, cells acquire specific functions, mediated by gene-environment interactions. Resting stem or progenitor cells are activated by molecules in their direct environment. These signals are transduced to the nucleus, where, depending on the nature of the signal(s), specific genes are activated, resulting in the production of RNA's (mRNA, non-coding RNAs) and proteins which trigger a differentiation program, that ultimately leads to formation of specialized, fully functional cells.

Although most somatic cells in a human body contain identical DNA sequences, the vast variety of cellular phenotypes implies that the information stored in the DNA sequence on its own is not sufficient to explain cell diversity and function. An additional layer of regulation, other than the DNA sequence itself, regulates accessibility of the DNA template. Nuclear DNA is not 'naked' but packed into a complex structure called chromatin. Chromatin predominantly consists of DNA wrapped around histone proteins. Histone proteins can be posttranslationally modified, and via these modifications many different protein complexes can bind and regulate chromatin. Chromatin structure is found in several 'states', ranging from tightly condensed inaccessible chromatin to open and accessible chromatin. Processes like DNA duplication (replication) and gene activation (transcription) are regulated by changes in chromatin structure, via increased or decreased accessibility of the DNA template. Collectively, these processes, that may involve concerted action of chemically modified DNA and proteins and non-coding RNAs, are referred to as epigenetic regulation. Polycomb group proteins (PcG) have been identified as epigenetic regulators of chromatin structure, and are best known as transcriptional regulators of Hox genes. An overview of our current understanding of regulation of replication and transcription by epigenetics in general and by PcG proteins in particular is presented in chapter 2.

Although insight into Polycomb biology has improved a lot over recent years, mechanisms of action of PcG proteins are far from fully understood. Main questions are What is the mechanism behind PcG-mediated transcriptional repression? Is there perhaps a role of PcG in replication and DNA repair? How are PcG proteins involved in mediating gene-environment interactions? How are PcG proteins regulated themselves? How are PcG proteins targeted to lineage-type specific gene targets?

As PcG proteins are involved in antero-posterior axis formation and many single PcG mutants cause skeletal abnormalities, this thesis aimed at elucidating the role of PcG proteins in one important aspect of enchondral ossification, namely chondrogenic differentiation.

Summary

The formation of bone (enchondral ossification) is a multistep process, in which an extracellular matrix is produced by chondrocytes that is ultimately calcified by osteocytes. Numerous locally produced soluble factors regulate different stages in this process, strongly suggesting that the microenvironment plays an important role in enchondral ossification. Many of these factors are activated in response to hypoxia, and downstream of HIF1 α activation, which is consistent with the absence of blood vessels in cartilage. Most of these findings result from *in vitro* studies, and currently studies on enchondral ossification often are performed on whole embryos or growth plates. Chapter 3 describes the molecular analysis of pathways relevant to enchondral ossification in a novel *in vivo* model in rabbits. The model makes use of experimentally induced local damage of periosteum, which results in activation of mesenchymal stem cell (MSC)-like activity, and *periosteal callus* formation. Subsequently the callus is used to functionally restore an experimentally induced articular cartilage lesion. Analysis of mRNA and protein expression in the repaired lesion confirms that the model recapitulates the sequential steps of normal chondrogenic development. In addition, we provide *in vivo* evidence that during the chondrogenic phase Hif1 α is activated, suggesting that the conditions of periosteal callus formation are, at least transiently, hypoxic.

During cellular differentiation cells receive extracellular signals that lead to changes of the transcriptome (all cellular RNAs produced by transcription of DNA), resulting in production of RNAs and proteins needed for a specific cell type to adapt and function. At the onset of this project knowledge on Immediate Early Genes (IEGs) in chondrogenic differentiation was limited. The identification of IEGs as strong responders to a changing microenvironment (inducing chondrogenic differentiation), prompted us to study the role of the IEG Egr1 during ATDC5 chondrogenesis (chapter 4). In a loss-of-function approach, using short hairpin RNAs targeting Egr1 mRNA, Egr1 was found to function in at least two, possibly interconnected, processes: Egr1 is required for transcriptional activation of downstream chondrogenic differentiation pathways and Egr1 controls initiation of crucial epigenome wide reprogramming during chondrogenesis. Additionally, Egr1-LOF cells accumulate DNA damage, activate DNA damage responses, and induce a senescence-like response, which may be related to abnormal epigenetic responses. Relevantly, chapter 4 describes two ways in which Egr1 may connect to PcG biology.

Polycomb repressive complex 1 (PRC1) mutant mice display abnormalities in skeletal development that correlate to defective maintenance of HOX gene expression boundaries, but our understanding of epigenetic mechanisms underlying chondrogenic differentiation is poor. To gain more insight in molecular mechanisms mediated by PRC1 during chondrogenesis, we studied Bmi1 in ATDC5 chondrogenic differentiation (chapter 5). Upon induction of differentiation ATDC5 cells undergo a transient phase of hyperproliferation. Loss of Bmi1 leaves the cells incapable of undergoing this expansion phase. Bmi1-LOF cells accumulate massive amounts of DNA damage,

arrest in S-phase, and consequently display characteristics of replicative senescence. Accumulation of DNA damage and activation of intra-S-phase checkpoints is associated with replication stress, collisions of replication and transcription machineries. This led us to study global localization of transcription and replication. Interestingly, control cells showed spatially separated regulation of these two DNA-based processes, whereas this coordinated regulation is lost in Bmi1-LOF cells during rapid proliferation. Furthermore, DNA damage in Bmi1-deficient cells accumulates at sites of *de novo* DNA synthesis, suggesting that loss of PRC1 function during hyperproliferation renders the cells unable to deal with increased chromatin stress. The data presented in chapter 5 reveals an important function for PRC1 in orchestrating simultaneous chromatin-associated processes, i.e. DNA replication and transcription, extending PRC1 function as merely transcriptional repressors.

Valproic acid (VPA) is a drug used to treat epilepsy and other neurological conditions. VPA administration during pregnancy can lead to neural tube defects, skeletal transformations and other adverse effects in the unborn child. VPA was designated as an epigenetic drug, but molecular mechanisms of actions are unclear. Treatment of differentiating ATDC5 cells with VPA in therapeutically relevant concentrations completely abrogates the ability of chondrogenic progenitors to differentiate, which is accompanied by severe alterations in global epigenetic modifications. Additionally, VPA affects the differentiation-induced hyperproliferation in a TP53-dependent manner. Completely in line with the data presented in chapters 4 and 5, VPA treated cells accumulate replication stress, DNA damage, and induce senescence-like responses, further strengthening the conclusions that epigenomic remodeling is critical for proper differentiation to occur (chapter 6).

Taken together, data in this thesis shows that in response to extracellular differentiation stimuli, epigenomic remodeling allows cells to and tune DNA-templated processes (transcription, replication), under conditions of increased proliferation and RNA and protein production. When epigenomic reprogramming fails, combined stress of uncoordinated transcription and replication stress leaves cells incapable of undergoing chondrogenic differentiation. The significance of the results in this thesis is discussed in chapter 7.

Samenvatting

Samenvatting

Alle genetische informatie die nodig is om een cel te laten functioneren ligt opgeslagen in 'n DNA. Genen kunnen afgeschreven worden in mRNA dat vervolgens kan worden vertaald naar eiwitten. Wanneer een cel zich voorbereidt voor celdeling, wordt het DNA in de celkern gedupliceerd en wordt het verdeeld tussen de twee dochtercellen, waardoor alle genetische informatie behouden blijft. Tijdens ontwikkeling, verkrijgen cellen een specifieke functie. Dit proces wordt gemedieerd door gen-omgevings interacties. Rustende stam of progenitor cellen worden geactiveerd door moleculen in hun directe omgeving. Deze signalen worden doorgegeven naar de celkern waar, afhankelijk van de aard van de signalen, specifieke genen worden geactiveerd, resulterend in de productie van RNA (mRNA, niet coderende RNA's) en eiwitten, die een differentiatie programma kunnen activeren, wat uiteindelijk leidt tot het ontstaan van gespecialiseerde, volledig functionele cellen. Hoewel bijna alle lichaamscellen in een menselijk lichaam identieke DNA sequenties bevat, impliceert de grote variatie aan gespecialiseerde cellen, dat de informatie die in het DNA ligt opgeslagen niet voldoende is om celdiversiteit en functie te verklaren. Een andere vorm van regulatie, naast de DNA sequentie zelf, reguleert toegankelijkheid van DNA. DNA in de kern is ingepakt in een complexe structuur, dat chromatine genoemd wordt. Chromatine bestaat voornamelijk uit DNA dat om histon eiwitten gewikkeld is. Histon eiwitten kunnen posttranslationeel gemodificeerd worden, en via deze modificaties kunnen vele verschillende eiwitcomplexen het chromatine binden en het reguleren. De structuur van chromatine bevindt zich in verschillende staten, variërend van een hechte, gecondenseerde, ontoegankelijke staat tot een open en toegankelijke staat. Cellulaire processen als DNA duplicatie (replicatie) en gen activatie (transcriptie) worden gereguleerd door veranderingen in chromatine structuur, via verhoogde of verlaagde toegankelijkheid van het DNA. Chromatine structuur wordt onder meer gereguleerd door chemische modificaties van DNA en eiwitten, en niet-coderende RNA. Samen wordt deze vorm van regulatie epigenetica genoemd. Polycomb groep eiwitten (PcG) zijn geïdentificeerd als epigenetische regulatoren van chromatine structuur, en zijn vooral bekend als transcriptionele regulatoren van Hox genen. Een overzicht van ons huidige begrip van regulatie van transcriptie en replicatie door epigenetica in het algemeen en specifiek door PcG eiwitten wordt beschreven in Hoofdstuk 2.

Hoewel het inzicht in Polycomb biologie flink is toegenomen de afgelopen jaren, worden de mechanismen van PcG functioneren nog maar voor een klein deel begrepen. Er zijn in dit vakgebied verschillende kernvragen. Wat is het mechanisme van PCG gemedieerde transcriptionele repressie? Is er wellicht een rol voor PcG in replicatie en DNA reparatie? Hoe zijn PcG eiwitten betrokken bij het mediëren van gen-omgevings interacties? Hoe worden PcG eiwitten zelf gereguleerd? Hoe worden PcG eiwitten naar celspecifieke genen gestuurd?

Aangezien PcG eiwitten betrokken zijn bij antero-posterior as vorming en PcG mutante muizen skelet afwijkingen hebben, richt deze thesis zich op het verhelderen van de rol van PcG eiwitten in een belangrijk aspect van enchondrale ossificatie, namelijk chondrogene differentiatie.

De vorming van bot (enchondrale ossificatie) is een meerstaps proces, waarin een extracellulaire matrix gevormd wordt door chondrocyten (kraakbeencellen), die uiteindelijk verkalkt wordt door osteocyten (botcellen). Talloze lokaal geproduceerde oplosbare factoren reguleren de verschillende stadia van deze processen, wat aangeeft dat de directe omgeving een sterke rol speelt in enchondrale ossificatie. Veel van deze factoren worden geactiveerd in reactie op een hypoxische omgeving, en activatie van de transcriptie factor HIF1 α , wat consistent is met de afwezigheid van bloedvaten in kraakbeen. Het overgrote deel van deze bevindingen komen voort uit *in vitro* studies. Daarnaast worden vele studies naar enchondrale ossificatie uitgevoerd op totale embryos of groeiplaten. Hoofdstuk 3 beschrijft de moleculaire analyse van relevante signaleringspaden tijdens enchondrale ossificatie in een nieuw *in vivo* model in konijnen. Dit model maakt gebruik van een lokaal geïnduceerde beschadiging van het periosteum, wat resulteert in de activatie van mesenchymale stem cell (MSC) gelijkende activiteit, en periosteale callusvorming. Vervolgens wordt de callus gebruikt voor een functioneel herstel van een experimenteel geïnduceerde articulaire kraakbeen lesie. Analyse van mRNA en eiwit expressie bevestigt dat het model de opvolgende stappen van normale chondrogene ontwikkeling recapituleert. Daarnaast, bieden we *in vivo* bewijs voor activatie van HIF1 α tijdens de chondrogene fase, wat suggereert dat de omstandigheden van periosteale callusvorming, op zijn minst transient, hypoxisch zijn.

Tijdens cellulaire differentiatie ontvangen cellen extracellulaire signalen die leiden tot veranderingen in het transcriptoom (alle RNA's die tijdens transcriptie van DNA geproduceerd worden), resulterend in productie van specifieke RNA's en eiwitten die een cel nodig heeft om zich aan te passen en een nieuwe functie aan te nemen. Aan het begin van dit project was er nauwelijks kennis van de rol van Immediate Early Genes (IEG's) tijdens chondrogene differentiatie. De ontdekking dat IEG's zeer sterk reageren op een veranderende omgeving, leidde tot het bestuderen van de rol van de IEG Egr1 tijdens chondrogene differentiatie in ATDC5 cellen (hoofdstuk 4). In een verlies van functie aanpak, gebruik makend van geïnduceerde afbraak van Egr1 mRNA, bleek Egr1 een tweeledige rol te spelen, die mogelijk verband met elkaar houden. Egr1 is vereist voor transcriptionele activatie van chondrogene differentiatie signaleringspaden, en ook initieert Egr1 cruciale epigenoom wijde herprogrammering tijdens chondrogenese. Daarnaast lopen cellen zonder Egr1 tijdens chondrogenese DNA schade op, activeren ze DNA schade responsen, en induceren ze een verouderingsresponse (senescentie), die vermoedelijk gerelateerd is aan abnormale epigenetische responsen. Hoofdstuk 4 beschrijft twee manieren waarin Egr1 vermoedelijk gelinkt aan PcG biologie is.

Polycomb repressive complex 1 (PRC1) mutante muizen vertonen abnormale skelet ontwikkeling, die correleert met defecte regulatie van Hox gene expressie, maar ons begrip van epigenetische mechanismen die hieraan ten grondslag liggen is beperkt. Om meer inzicht te krijgen in de moleculaire mechanismen die door PRC1 gemedieerd worden tijdens chondrogenese, bestudeerden we Bmi1 in ATDC5 chondrogene differentiatie (hoofdstuk 5). In response op inductie van differentiatie ondergaan ATDC5 cellen een transiente fase van hyperproliferatie. Verlies van Bmi1 functie leidt ertoe dat de cellen deze expansie fase niet kunnen ondergaan. Zonder Bmi1 lopen de cellen enorme hoeveelheden DNA schade op, ondergaan ze een S-fase arrest, en laten ze vervolgens karakteristiekeken zien van replicatieve senescentie. Opstapeling van DNA schade en activatie van een S-fase arrest wordt geassocieerd met replicatie stress, collisies van replicatie en transcriptie machineriën. Hierom bestudeerden we globale lokalisatie van transcriptie en replicatie. Controle cellen lieten een ruimtelijke scheiding zien van deze twee processen, terwijl deze gecoördineerde regulatie verloren is gegaan in cellen zonder Bmi1, die deze fase van hyperproliferatie ondergaan. Daarnaast lopen de Bmi1 deficiënte cellen DNA schade op op posities waar replicatie plaatsvindt, wat suggereert dat cellen met verlies van PRC1 functie tijdens hyperproliferatie niet in staat zijn om met verhoogde chromatin stress om te gaan. De data in hoofdstuk 5 laat zien dat PRC1 een belangrijke rol speelt in het regisseren van chromatine geassocieerde processen, zoals replicatie en transcriptie, wat betekent dat PRC1 niet enkel functioneert als transcriptionele repressor.

Valproaat is (VPA) is een medicijn dat wordt gebruikt om epilepsie en andere neurologische aandoeningen te behandelen. VPA gebruik tijdens zwangerschap kan leiden tot neurale buis defecten, malformaties van het skelet, en andere aandoeningen bij het ongeboren kind. VPA is beschreven als een epigenetisch medicijn, maar de moleculaire effecten zijn onbekend. Behandeling van differentierende ATDC5 cellen met therapeutisch relevante concentraties VPA leidt ertoe dat ATDC5 cellen het vermogen om te differentieren verliezen. Dit gaat gepaard met zeer sterke veranderingen in globale epigenetische modificaties. Daarnaast, verliezen deze cellen de capaciteit om te hyperproliferen in een TP53 afhankelijke manier. Totaal in lijn met de experimenten beschreven in hoofdstukken 4 en 5, lopen de VPA behandelde cellen replicatie stress en DNA schade op. Eveneens activeren deze cellen senescentie responsen, wat verder bijdraagt aan de conclusies dat epigenomische herprogrammering essentieel is om volledige differentiatie plaats te laten vinden (hoofdstuk 6).

De data in dit proefschrift laat zien dat in response op extracellulaire differentiatie stimuli, epigenomische herprogrammering de cellen in staat stelt om DNA gebaseerde processen (o.a. transcriptie en replicatie) op elkaar af te stemmen tijdens omstandigheden van versnelde proliferatie, en RNA en eiwit productie. Wanneer epigenomische herprogrammering niet goed plaats vindt, verliezen cellen het vermogen om chondrogeen te differentiëren, vanwege replicatie stress. De significatie van de resultaten beschreven in dit proefschrift worden bediscussieerd in hoofdstuk 7.

Dankwoord

Dankwoord

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Curriculum Vitae

Frank Spaapen werd geboren op 7 februari 1978 te Haarlem en groeide op in Vogelenzang. In 1990 rondde hij het VWO af op het Sancta Maria te Haarlem. In datzelfde jaar begon hij de studie Gezondheidswetenschappen aan de universiteit te Maastricht. Na gekozen te hebben voor het moleculair biologisch traject, voerde hij een korte stage uit op de afdeling Moleculaire Celbiologie onder supervisie van Bert Schutte. Tijdens deze stage werd klieving van cytokeratines tijdens inductie van apoptose bestudeerd. Tijdens zijn afstudeerstage aan de afdeling Gezondheidsrisico analyse en toxicologie werkte hij aan studies naar de genotoxische effecten van carcinogenen in humane longepitheelcellen. Deze stage was onder begeleiding van Prof. Dr. F.J. van Schooten en Danielle Curfs, en werd met succes afgerond waardoor hij in februari 2003 zijn diploma mocht ontvangen. Direct aansluitend op het afstuderen begon hij zijn werkzaamheden in de onderzoeksgroep van Dr. J.W. Voncken in de capaciteitsgroep Moleculaire Genetica aan de Universiteit Maastricht waar zijn interesse in moleculaire biologie zeer goed van pas kwam. Na een aantal maanden als analyst te hebben gewerkt, begon hij zijn promotieonderzoek naar de rol van PcG eiwitten tijdens chondrogene differentiatie. Na het afronden van het promotieonderzoek blijft hij werkzaam in de onderzoeksgroep van Dr. J.W. Voncken waar hij zich richt op de rol van PcG eiwitten tijdens replicatie timing. Door de toekenning van een EMBO fellowship wordt hij in staat gesteld om bekend te raken met de technieken om replicatie timing te bestuderen onder begeleiding van Dr. V. Azuara op het Imperial College te Londen.

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Abbreviations

Abbreviations

ACAN	Aggrecan
AP	Antero-posterior
ATM	Ataxia telangiectasia mutated
ATR	Ataxia telangiectasia mutated- and Rad3-related
BARR	Barren
BMI1	B-cell-specific Moloney murine leukemia virus integration site 1
BMP	Bone morphogenetic protein
BrdU	5-bromo-2-deoxyuridine
CBX	Chromobox protein
ChIP	Chromatin immunoprecipitation
Chk	Checkpoint kinase
Col10a1	Collagen 10a1
Col2a1	Collagen 2a1
CRM	Cramped
CryGS	γ Crystalline
DAPI	4',6-diamidino-2-phenylindole
DDR	DNA Damage Response
DNMTs	DNA methyltransferases
DSB	Double strand break
DUB	Deubiquitylase
e.g.	Exempli gratia
ECM	Extracellular matrix
EDR	Early developmental regulator
Egr	Early growth response
ERSS	Endoplasmatic reticulum stress signaling
ESCs	Embryonal stem cells
ETP	Enhancer of Trithorax and Polycomb
ETP	Etoposide
EZH2	Enhancer of Zeste homologue
FC	Fold change
FDA	Food and Drug Administration
GMNN	Geminin
GTF	General transcription factor
H2A	Histone 2A
H2AX	Histone 2Ax
H3K27me3	Histone 3 lysine 27 trimethylation
H3K4	Histone 3 lysine 4
H3K9me3	Histone 3 lysine 9 trimethylation
H3S10	Histone 3 serine 10
H3S28	Histone 3 serine 28
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HIF1 α	Hypoxia inducible factor 1 α
HKMT	Histone lysine methyltransferase
HMT	Histone methyltransferase
HZ	Hypertrophic zone
i.a.	Inter alia
i.e.	id est
IEG	Immediate early gene
Ihh	Indian hedgehog
Kap1	KRAB-associated protein1
KD	Knockdown
LCR	Locus control region
LOF	Loss of function
MAPK	Mitogen activated protein kinase
MCM	Mini chromosome maintenance

MSC	Mesenchymal stem cell
NSC	Neuronal stem cell
OIS	Oncogene induced senescence
ORC	Origin recognition complex
PC	Polycomb
PC	Perioseal callus
PcG	Polycomb Group
PCNA	Proliferating cell nuclear antigen
PH	Polyhomeotic
PHC	Polyhomeotic-like
PIC	Pre-initiation complex
PID	Post induction of differentiation
PO	Postoperative
POSTN	Periostin
PRC	Polycomb repressive complex
PRE	Polycomb responsive element
pre-RC	pre-Recognition complex
PTHrP	Parathyroid related protein
PTM	Posttranslational modification
RING	Really interesting new gene
RNF2	Ring finger protein
ROS	Reactive oxygen species
RPOLII	RNA polymerase II
SAHF	Senescence associated heterochromatin formation
SASP	Senescence associated secretory phenotype
SA β Gal	Senescence associated β galactosidase
SCMH	Sexcombs on midleg homologue
shRNA	Short hairpin RNA
siRNA	Small interfering RNA
SUZ12	Suppressor of Zeste 12
TA	transit amplification
TBP	TATA-box binding protein
TFIIA	Transcription factor IIA
TFIIB	Transcription factor IIB
TFIID	Transcription factor IID
TGF β	Transformation growth factor β
Topo2	Topoisomerase 2
TRX	Trithorax
UPR	Unfolded protein response
VEGF	Vascular endothelial growth factor
VPA	Valproic acid
WT	Wildtype

Colour pictures

Fig.3.2 Collagen Type II in periosteal reactive tissue (callus)

Fig. 3.3 Periostin/POSTN expression in periosteum

Fig 3.4 BMP-4 expression in periosteal callus

Fig 4.3 Egr1 is required for proper replication during chondrogenic replication

Fig 4.4c. Egr1-LOF accumulate DNA damage

Fig 4.5g. Global histone modifications are affected in Egr1-LOF cells

Fig 5.1c Bmi1 expression

Fig 5.2e BrdU staining

Fig 5.2d BrdU and H3S10ph staining

Figure 5.3. ?H2Ax and BrdU staining in ATDC5 (A,B) and human primary chondrocytes (D).

Fig. 5.4 D,E Abnormal staining of histone modifications

Fig. 5.4 C, F Senescence staining ATDC5 cells and ribcage of wildtype mice

Figure 5.6. Accumulation of DNA damage at sites of deregulated replication and transcription.

Figure 5.7. IF analysis of Bmi1, Phc1, Geminin and γH2A.X in ATDC5 shControl and shBmi1.

Figure 5.S3. Accumulation of DNA damage in shBmi1 cells.

Figure 5.S4. Bmi1-LOF results in DNA damage responses and a proliferative arrest

Figure 5.S4 continued. Bmi1-LOF results in DNA damage responses and a proliferative arrest

Fig. 5.S5D. Senescence during chondrogenesis

Fig. 5.S6 A,B Senescence staining on longitudinal bones ribcage/spinal column of wild type mice

Fig 5.S8. IF staining for PCNA BrdU and pRPOL2 in ATDC5 shControl and shBmi1 at 2d pid.