The Role of Nucleotide Excision Repair (NER) Factors in Genomic Imprinting

A thesis written

by

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Ο ρόλος του μονοπατιού εκτομής νουκλεοτιδίων (NER) στη γονιδιακή αποτύπωση

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Το τμήμα Βιολογίας
σε μερική εκπλήρωση των απαιτήσεων
για το βαθμό του
Μεταπτυχιακού τίτλου
στο γνωστικό αντικείμενο της

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ABSTRUCT

The genome is constantly challenged by endogenous and exogenous DNA damaging factors. To meet this challenge, cells have evolved a plethora of mechanisms that counteract the damaging threat. The nucleotide excision repair (NER) pathway is responsible for removing primarily bulky helix-distorting damage, such as the UV-induced lesions.

Defects in members of the NER pathway are associated with developmental abnormalities, besides cancer and ageing. Recent work demonstrates the function of several NER factors in processes beside DNA repair.

In this study, we focus on the ERCC1 protein which binds the XPF endonuclease creating a heterodimer in the NER pathway, responsible for the 5' incision of the damaged strand. The fact that ERCC1-XPF is involved in DNA demethylation and *Ercc1*$^{-/-}$ mice exhibit developmental defects prompted us to examine the effects of ERCC1 in genomic imprinting. As it is well known, the molecular mechanism that is responsible for appropriate expression of the imprinted genes, which are essential for proper development of an organism, is governed by DNA methylation marks.

Περίληψη

Όπως είναι γνωστό, ο μοριακός μηχανισμός που είναι υπεύθυνος για την κατάλληλη έκφραση των αποτυπωμένων γονιδίων, τα οποία είναι απαραίτητα για τη σωστή ανάπτυξη ενός οργανισμού, διέπεται από τα σήματα μεθυλίωσης του DNA.

Το γονιδίωμα των κυττάρων εκτείθεται καθημερινά τόσο σε ενδογενείς όσο και εξωγενείς παράγοντες, που μπορούν να προκαλέσουν βλάβες στο DNA. Για να ανταπεξέλθουν σε αυτή την πρόκληση, τα κύτταρα έχουν εξελίξει μια πληθώρα μηχανισμών που επιδιορθώνουν βλάβες στο DNA. Το μονοπάτι επιδιόρθωσης εκτωμής νουκλεοτιδίων (NER) είναι υπεύθυνο για την απομάκρυνση βλαβών που προκαλούνται κυρίως από την υπεριώδη ακτινοβολία.

Βλάβες σε προτείνες του μονοπατιού NER σχετίζεται με αναπτυξιακές ανωμαλίες, πέρα από την εμφάνιση καρκίνου και τη γήρανση. Πρόσφατες μελέτες δείχνουν την λειτουργία των NER παραγόντων σε διαδικασίες πέρα από την επιδιόρθωση του DNA.

Σε αυτή τη μελέτη, θα επικεντρωθούμε στην πρωτείνη ERCC1, η οποία δεσμεύει την ενδονουκλεάση XPF σχηματίζοντας ένα ετεροδιμερές στο μονοπάτι του NER, το οποίο είναι υπεύθυνο για την τομή στο 5’ άκρο της αλληλουχίας που φέρει την βλάβη. Το γεγονός ότι η ERCC1-XPF εμπλέκεται στην απομεθυλίωση του DNA και τα *Ercc1*$^{-/-}$ ποντίκια φέρουν αναπτυξιακές ανωμαλίες μας ώθησε να εξετάσουμε την επίδραση της ERCC1 στην γονιδιακή αποτύπωση. Όπως είναι γνωστό, ο μοριακός μηχανισμός που είναι υπεύθυνος για την κατάλληλη έκφραση των αποτυπωμένων γονιδίων, τα οποία είναι απαραίτητα για τη σωστή ανάπτυξη ενός οργανισμού, διέπεται από τη μεθυλίωση ρυθμιστικών περιοχών του DNA.

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INTRODUCTION

1. NER mechanism and the factors involved

A) The DNA Damage Problem

Preservation of genetic information is of prime importance to all living systems. However, the integrity of the genome is continuously threatened by a variety of environmental and endogenous agents that damage the DNA, as well as by intrinsic instability of chemical bonds in DNA itself. Oxidative stress, X-rays, ultraviolet (UV) light and numerous chemicals induce a wide mélange of lesions in DNA. Obviously, this affects the proper functioning of vital DNA-metabolizing transactions. Immediate effects of DNA damage include a physical block of transcription and replication. As DNA lesions interfere with the process of transcription, they also affect gene expression and consequently vital responses for the survival of a cell against hazardous threats that could also lead to cell death (Garinis et al., 2006; Garinis et al., 2005; Jans et al., 2006). Long-term effects of DNA damage involve induction of mutations via replication of damaged DNA, which ultimately provides a major initiating and driving step in the process of carcinogenesis. A substantial body of evidence argues that DNA damage and mutations accumulate with age in mammalian cells (Vijg, 2000). However, one should distinguish between mutations in the genome and the DNA damage itself. Whereas DNA damage is an undesired chemical alteration in the base, sugar or phosphate that alters the properties of DNA, mutations represent fixed errors in the coding sequence of otherwise chemically unaffected DNA (Hoeijmakers, 2007). Eventually, the effects of distinct types of lesions diverge with respect to helix distortion, ability to pause or obstruct DNA replication, block ongoing transcription or else hamper the battery of repair systems and other caretakers that continuously safeguard the genome. Mutagenic lesions (i.e. spontaneous deaminations, depurinations and certain oxidized bases) are predominantly responsible for mutations and contribute to carcinogenesis. On the other hand, cytotoxic or cytostatic lesions (e.g. double strand breaks, UV-induced lesions, DNA interstrand cross links [ICLs], uncapped telomeres and certain oxidized bases) are thought to predominantly cause apoptosis or senescence, respectively, thereby contributing to aging (Mitchell et al., 2003). That being said, however, mutagenic lesions can become cytotoxic (in case the damage is excessive) whereas upon faulty repair, cytotoxic lesions may also turn into mutagenic ones.

This simplified distinction between cytotoxic/cytostatic and mutagenic lesions appears to support, to some extent, the notion that DNA repair systems that primarily patch mutagenic lesions prevent cancer while repair pathways that primarily attack cytotoxic lesions combat aging. Either way, the strong cancer predisposition observed in certain inherited human disorders with malfunctioning genome care-taking systems (i.e. Xeroderma pigmentosum [XP], Li-Fraumeni, hereditary non-polyposis colorectal cancer, ataxia telangiectasia [AT] as well as the increasing number of progeroid syndromes with defects in DNA repair (e.g. Cockayne syndrome [CS], trichothiodystrophy [TTD] or XPF-ERCC1 [XFE] syndrome) emphasize the biological impact of genome care-taking mechanisms in both cancer and aging (Bootsma, 1998; de Boer et al., 2002; Friedberg E., 1995; Niedernhofer et al., 2006; van der Pluijm et al., 2006).

B) Nucleotide Excision Repair (NER): at the Crossroad of Cancer and Aging

To withstand the harmful effects of (persisting) DNA lesions, cells are equipped with a set of complementary repair pathways with specific, (partially) overlapping substrate specificity and control mechanisms that arrest cell cycle progression, thereby providing a time window for repair (Friedberg E., 1995; Hoeijmakers, 2001). Base excision repair (BER) or nucleotide excision repair (NER) and its subpathways are predominantly employed to repair DNA lesions that affect only one DNA strand. However, although BER has a vital role in the repair of oxidative lesions, mutations in genes associated with this pathway are either lethal or, when redundant, confer no obvious phenotypes. By contrast, mutations in distinct NER factors lead to premature aging or increased cancer predisposition (Andressoo et al., 2006; Mitchell et al., 2003). NER responds to a bewildering range of lesions that distort the helical DNA structure, via the concerted action of 25 or so proteins that sequentially execute damage recognition, chromatin remodeling, local opening of the DNA double helix, incision of the damaged DNA strand on both sides of the lesion, excision of the 27-29mer oligonucleotide containing the damage, and gap-filling DNA synthesis followed by strand ligation (de Laat et al., 1999; Friedberg E., 1995; Hoeijmakers, 1994; Wood, 1996).

Two sub-pathways of NER can be distinguished that differ primarily in how the damage is initially recognized: the global genome repair (GGR) sub-pathway is responsible for the removal of lesions from the entire genome (Figure 1). A major limitation of this system, however, is that certain types of damage (like UV-induced CPDs) are less well recognized

and accordingly less efficiently repaired. To avoid that such lesions hamper transcription by stalling RNA polymerase II, a distinct NER subpathway has evolved, called Transcription-Coupled Repair (TCR). This system directs the repair machinery preferentially to the template strand of actively transcribed DNA and operates as a fast backup system for lesions that are slowly repaired by GGR (Figure 1). In man, the clinical consequences of defective NER are illustrated by the phenotype of four rare, autosomal recessive disorders: xeroderma pigmentosum (XP; affected proteins: XPA-XPG), Cockayne syndrome (CS; affected proteins: CSB, CSA), trichothiodystrophy (TTD; affected proteins: XPB, XPD, TTDA) and XPF-ERCC1 syndrome (XFE; affected proteins: XPF, ERCC1) (Bootsma, 2001; Giglia-Mari et al., 2004; Jaspers et al., 2007; Niedernhofer et al., 2006). The common hallmark of these pleiotropic disorders is pronounced hypersensitivity to solar (UV) light. With the exception of XPC and XPE, where only the GGR subpathway of NER is affected, most XP patients are defective in both GGR and TCR and suffer from pigmentation anomalies and a 2000-fold elevated risk of developing skin cancer in sun-exposed areas of the body, often in combination with progressive neurological degeneration. CS patients are defective in the TCR subpathway of NER and present with cachexia, dwarfism, neurological abnormalities, impaired sexual development, kyphosis, osteoporosis and severely reduced lifespan (mean age of death: 12.5 years) (Bootsma et al., 2002; Nance and Berry, 1992). TTD patients are partially defective in TCR, as well as GGR, and share the symptoms associated with CS. The only patient documented so far with XPF-ERCC1 syndrome carries a TCR defect as well as a defect in the repair of DNA interstrand crosslinks (Jaspers et al., 2007). Many of the CS and TTD features are progressive and resemble premature aging. However, as most of these patients develop some, but not all, aspects of normal aging in an accelerated manner, CS, TTD or XPF-ERRC1 are considered “segmental progeroid syndromes” (Martin, 2005). In essence, congenital defects in TCR can lead to premature aging syndromes (CS, TTD) but show no cancer predisposition. Instead, defects in GGR may give rise to disorders with greatly elevated cancer rates (e.g. XP) but no progeria (Figure 1).

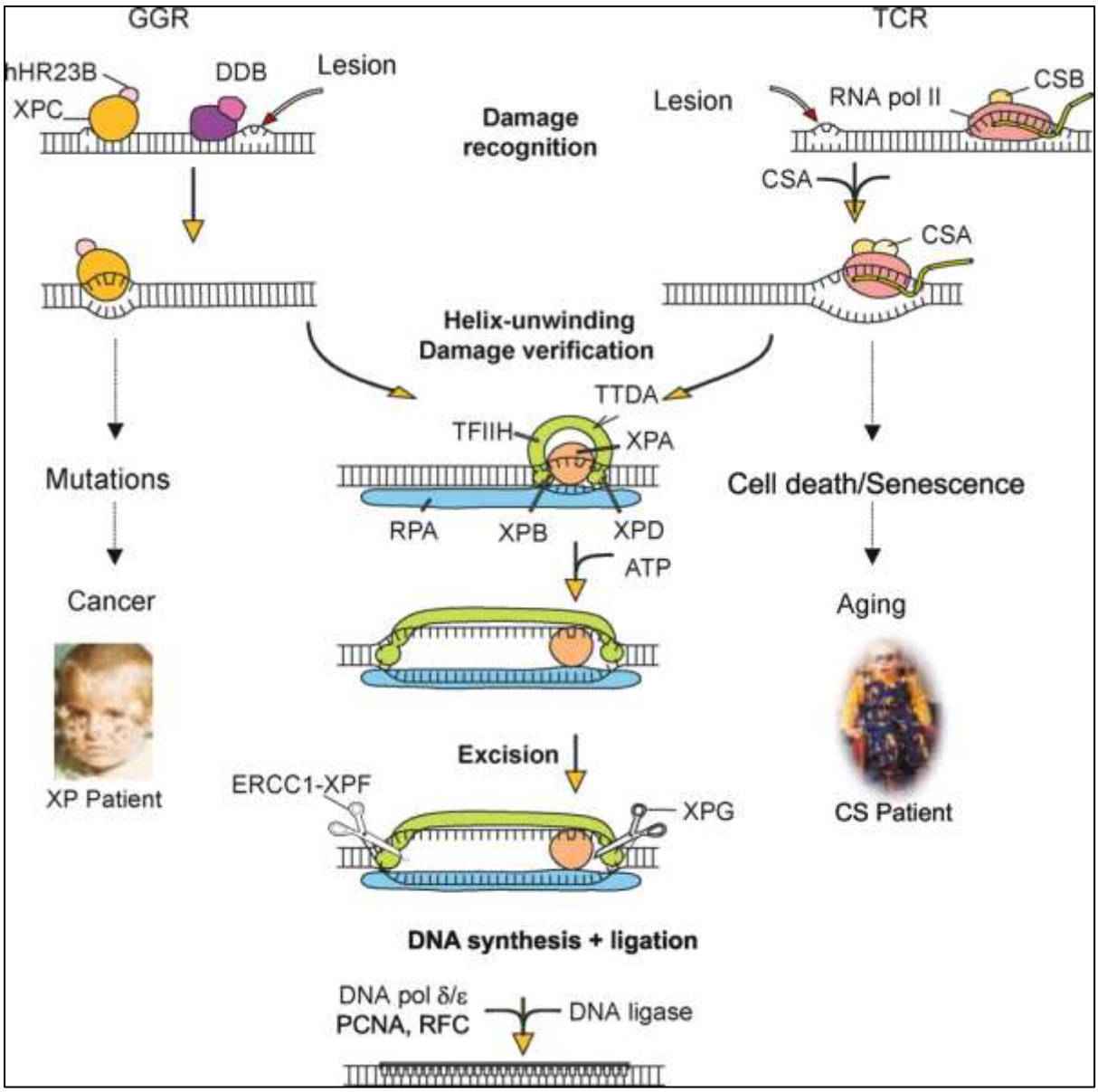


Figure 1. The nucleotide excision repair (NER) pathway. The NER pathway can be distinguished in two sub-pathways. Global genome NER (GGR) recognizes and removes helical distortions throughout the genome, while Transcription-coupled repair (TCR) selectively recognizes and removes helical distortions from the transcribed strand of active genes. The two sub-pathways of NER differ primarily in how the damage is initially recognized. Following damage detection, GGR and TCR merge into a common mechanism to unwind the DNA around the lesion, to stabilize and excise the DNA fragment containing the damage, and to fill in and ligate the single strand gap.

2. NER defects in development: the importance for man

A) Mouse Models for NER Syndromes

At present, a comprehensive series of mouse mutants are available with defects in NER that show either progeria or increased cancer predisposition. For instance, mice with a homozygous point mutation in the *Xpd* gene, a DNA helicase that functions in both repair and transcription, recapitulate most of the features seen in TTD patients (de Boer et al., 1998), including brittle hair, osteoporosis, osteosclerosis, kyphosis, cachexia and a reduced lifespan (de Boer et al., 2002). These findings provided the first substantial evidence that aging in TTD mice is likely caused by unrepaired DNA damage compromising transcription that leads to functional inactivation of critical genes and consequently to age-related pathology. Similarly, mice with defects in *Csa* and *Csb* genes consistently mimic the sensitivity of CS patients to solar (UV) irradiation and show accelerated photoreceptor loss (Gorgels et al., 2007), reduced body weight, and mild neurodegeneration (van der Horst et al., 2002; van der Horst et al., 1997). Notably, complete NER inactivation (by concurrent inactivation of the *Xpa* gene) substantially enhances the severity of CS features of TCR-compromised TTD or CSB mice. For instance, $Xpd^{TTD/TTD}/Xpa^{-/-}$ double mutant animals display dramatic postnatal growth attenuation, kyphosis, ataxia, abnormal locomotor activity, as well as progressive weight loss and died prematurely before weaning (de Boer et al., 2002). In a similar fashion, newborn $Csb^{m/m}/Xpa^{-/-}$ mice exhibit very similar progeroid features and, like the $Xpd^{TTD/TTD}/Xpa^{-/-}$, die at ~one month of age (van der Pluijm et al., 2006). These findings put forward the notion that an increase in the total DNA-damage load on the transcribed strand of active genes likely underlies the cytotoxicity and dramatic progeria seen in TCR-deficient animals. Interestingly, the double-mutant mice ($Xpd^{TTD/TTD}$/Xpa$^{-/-}$ or $Csb^{m/m}/Xpa^{-/-}$) are both defective in TCR and GGR. Even so, these mice only show greatly accelerated ageing but no enhanced cancer predisposition. This indicates that either the effect of cytotoxic/cytostatic lesions override mutagenic lesions when the repair of both is compromised as cells might undergo apoptosis before DNA lesions can result in mutations, or else that tumors need considerably more time to develop than the extremely short lifespan of double mutant animals. However, the latter seems rather unlikely as TTD mice have relatively long lifespans (~1.5 years), yet they develop cancer less frequently than wild-type littermates. The recent discovery of a novel syndrome, designated XFE (XPF-ERCC1) progeria (Niedernhofer et al., 2006) has further expanded the spectrum of lesions that can interfere with DNA metabolism and

lead to progeria. *Ercc1*$^{-/-}$ mice (carrying a defect in an endonuclease required for NER as well as for repair of cytotoxic DNA interstrand cross-links) mimic the XPF-ERCC1 syndrome and demonstrate most of the progeroid features described above but also others that are, in part, distinct from mice only deficient TCR such as dramatic liver, kidney and bone marrow pathology (Niedernhofer et al., 2006). Hence, different types of lesions and repair systems might differentially obstruct vital biological processes such as transcription and/or replication leading preferentially to cell death or senescence of particular cell types, tissues or organs and ultimately to "segments" of "age-related deterioration" over time (de Boer and Hoeijmakers, 2000; Hasty et al., 2003; Mitchell et al., 2003).

3. The importance of imprinting during development

A) Parental Genomic Imprinting

Genomic imprinting is an epigenetically regulated process that controls the monoallelic expression of distinct genes in a parent of origin-specific manner (Fergusone-Smith, 2011). Imprinted genes are found in a small number within the genome, and comprise nearly one per cent of mammalian genes. In particular, the mouse genome comprises about 144 imprinted genes (http://www.mousebook.org/catalog.php?catalog=imprinting). The great majority of imprinted genes are not distributed evenly across the mammalian genome; instead, they are found clustered in chromosomal domains (Edwards CA, Ferguson-Smith AC., 2007). It has been shown that appropriate expression of the imprinted genes is important for normal development of an organism.

Based on available data for imprinted genes across genomes, parental genomic imprinting appears to prevail predominantly in therian mammals, including the placental and the marsupial animals. However, not all therian mammals show the same degree of imprinted genes; for instance, in primates the Igf2r gene is not imprinted (Kalscheuer et al., 1993).

B) Imprint – The DNA Methylation Mark

The imprinted DNA sequences are marked with their parental origin to obtain a correct allele specific expression pattern. The parental specific mark has to fulfil three main criteria. First, it must be able to influence transcription. Second, after every cell division the mark must be inherited by the same parental allele. Third, the mark must be erasable in order for the cell to

be able to remark the allele according to the parental origin during gametogenesis. A common feature of the imprinted regions is the allele specific DNA methylation mark. So far, only DNA methylation has been clearly shown to function as the gametic imprint for imprinted genes, and to date, DNA methylation represents the only known heritable modification. Thus, there is only one parental allele that is methylated while the remaining allele is not.

The imprints of the paternal mouse germ cells are set up in embryonic day E15.5 - 17.5. At this stage, the germ cells are called spermatogonia, and the imprints are maintained throughout life. By contrast, the maternal imprints of the mouse oocyte are established postnatally at about P10 – 25 days of age, which are also maintained throughout life (Figure 2). The genome undergoes two phases of DNA demethylation. The first phase of genome-wide DNA demethylation occurs in the primordial germ cells (PGCs), while the cells migrate to the genital ridge at about 10 – 12 embryonic days. At this first phase of DNA demethylation the imprints are not maintained and get erased. In this way the germ cell is able to set up its parent of origin specific mark. The second genome-wide DNA demethylation phase occurs after fertilization during the formation of the zygote (Figure 2). It has been shown that the imprints are not erased but are maintained at this stage of demethylation (Abramowitz and Bartolomei, 2011). De novo DNA methylation occurs a few days later during development at the implantation stage.

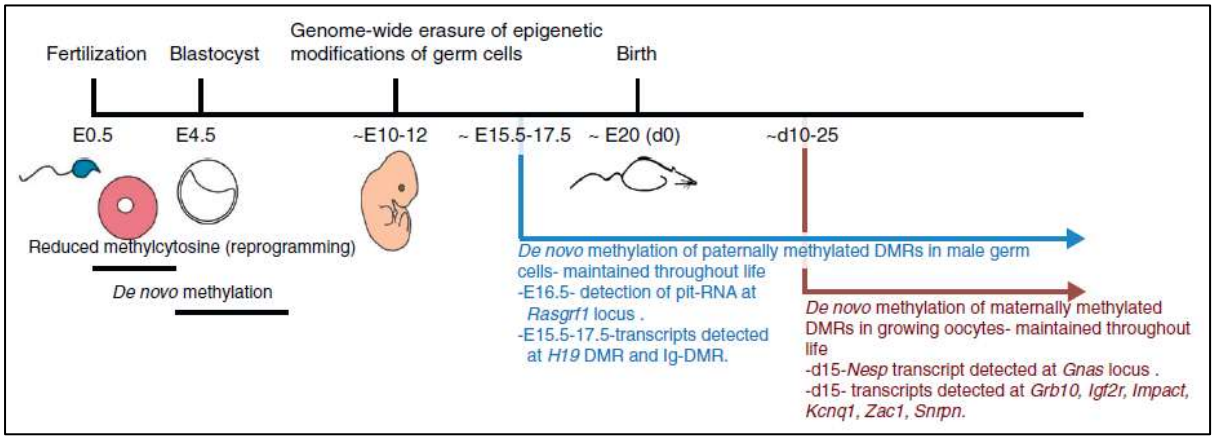


Figure 2. Epigenetic reprogramming of DNA methylation in the mouse genome.

C) Differentially Methylated Regions Control the Imprinting of Genes

The region where the imprint is established is called differentially methylated region (DMR). The DMRs are methylated by the de novo DNA methyltransferases (DNMTs). With the exception of Rasgrf1 DMR which requires DNMT3a and DNMT3b, all other DMRs require DNMT3a (Kato et al., 2007). DMRs can be distinguished into primary and secondary genomic structures. Primary DMRs acquire gamete-specific methylation during gametogenesis, and maintain the allelic methylation differences throughout development. Instead, secondary DMRs are established after fertilization which is often a consequence of imprinted expression. In addition to the two above mentioned DNA methyltransferases, DNMT3L which lacks methyltransferase activity plays a crucial indirect role in the methylation of the DMRs. DNMT3L may bind unmethylated histones (H3K4), and is absent in methylated (H3K4me3) ones (Ooi et al., 2007). DNMT3L may also interact with DNMT3a and DNMT3b, and in the absence of DNMT3L, hypomethylation of the DMRs is observed. Thus, DNA methylation patterns of the DMRs are likely governed by PTM patterns established on the nucleosomes.

D) Mechanism of Genomic Imprinting

Delineating how the imprinted genes are regulated is of fundamental importance. To date, two main classes of cis-acting silencing mechanisms can be applied on the imprinted gene clusters studied so far.

The “insulator model”, suggests that an insulator molecule acts as a boundary element preventing the proximity between the enhancer and the promoter (Wan and Bartolomei, 2008). The best studied imprinted locus where the insulator model applies is the Igf2-H19 locus (Smits et al., 2008). In this locus, the paternally expressed Igf2 gene, and the maternally expressed H19 gene share enhancers. The insulator, in this case the zing-finger protein CCCTC-binding factor (CTCF), binds on the DMR also called ICR (imprinted control region) which is located between the two genes about 2 Kbs upstream of H19. On the maternal allele, the ICR is not methylated allowing CTCF to bind, thereby preventing the Igf2 to come in contact with the shared enhancers (Figure 3). On the other hand, the ICR on the paternal allele is methylated, thereby CTCF cannot bind, and the enhancers are able to access Igf2. In addition, the absence of CTCF from the paternal ICR is required for the methylation at the H19 promoter and silencing of H19.

The "non-coding RNA (ncRNA) mediated silencing" model (Wan and Bartolomei, 2008) suggests that the ICR includes a differentially methylated promoter that regulates the expression of an ncRNA. In case where the ICR is not methylated, the ncRNA is expressed which in turn is able to repress its target genes. By contrast, if the ICR is methylated the ncRNA is not expressed, and thus it is not able to repress its target genes.

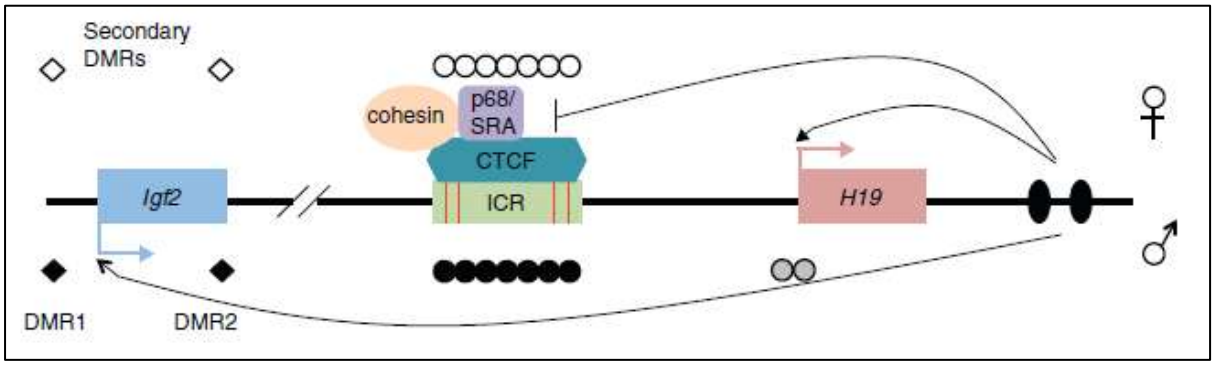


Figure 3. The Igf2-H19 imprinted locus. Enchancers: black ovals; Unmethylated sites: open circles and diamonds; Methylated sites: black circles and diamonds; Paternal methylated H19 promoter: grey circles

4. Defective imprinted genes and growth defects during development

A) Perturbed Imprinting in Pre-natal and Post-natal Development

Normal mammalian development requires the appropriate expression of imprinted genes. Changes in the expression levels of imprinted genes are often associated with functional consequences leading to developmental defects. For instance, many human and mouse imprinted genes are known to be dosage-sensitive and essential for prenatal growth, for normal brain function, and postnatal energy homeostasis. In general, the expression levels of imprinted genes are high during the prenatal stages whereas these are predominantly down-regulated postnatally.

Early in development, resources are controlled at the interface between mother and foetus in a way that is partially regulated by imprinted genes within the placenta. In particular, perturbed imprinted expression of the gene Igf2 in the placenta impairs nutrient transport to the growing foetus resulting in defective placental development (Sibley et al., 2004).

Moreover, appropriate expression of the Ascl2 imprinted gene, is required for normal differentiation of spongiotrophoblast cells during the early stages of the developing murine placenta (Guillemot et al., 1995). Proper growth of the embryo is not only affected by perturbed imprinting in the placenta. For example, overexpression of the imprinted gene Dlk1, results in a growth enhanced foetus even when the placenta expresses normal levels of the gene (da Rocha et al., 2009).

The genes that are imprinted in the brain are primarily involved in biological processes that modulate the metabolic axes and the behaviour which is controlled primarily by the nervous system and the endocrine system, and learning. Prader-Willi syndrome (PWS) and the Angelman syndrome (AS) represent two disorders with perturbed imprinting in the brain. Although the two disorders are phenotypically distinct from each other, the genes affected are mapped in the same imprinted locus in the human genome (Horsthemke and Wagstaff, 2008).

Imprinted genes do not only function in the placenta and the brain, but also influence the development of other organ systems. For example, Dlk1 acts as a ligand in the Notch signalling pathway, which regulates and controls multiple cell differentiation processes during embryonic and post-natal development (Moon et al., 2002; Raghunandan et al., 2008). Moreover, it has been shown that perturbed imprinting of Cdkn1c which is highly expressed in many somatic tissues during development, and plays crucial role of the cell cycle, leads to defects in organ size (Jia et al., 2007b).

5. The importance of DNA demethylation and its impact on gene expression

A) DNA Methylation

Methylation in the mammalian genome occurs on the cytosine nucleotide, most commonly at 5'-CG-3' dinucleotides. Depending on whether the methyl group is placed (cytosine residue is methylated) on one or both strands, the DNA is refered to as hemi-methylated and homo-methylated respectively.

DNA methylation is an epigenetic modification and although it does not alter the primary DNA sequence it is able to influence normal development, gene expression patterns, and genomic stability. The DNA methylation patterns can be very dynamic as can be seen by

genome-wide demethylation and methylation status during the early stages of mammalian development.

B) DNA Methylation affects Transcription

The fact that DNA methylation is active involved in regulating gene transcription and thereby affecting the expression of a gene is well established. It has been shown that methylation sensitive CpG regions are able to interact direct with transcription factors, methyl- CpG binding proteins and several other factors that may alter chromatin structure. How exactly proteins with a methyl- CpG binding domain (MBD) repress transcription is not clear yet. The best known mechanism involves the recruitment of two co- repressor complexes Sin3 and Mi-2/NuRD by a MBD protein to methylated DNA. The co- repressors in turn recruit a core histone deacetylase complex. The removal of the acetyl groups of the histones (H3 and H4) results in a more condense chromatin affecting the binding of transcription factors. Besides the deacetylase activity Mi-2/NuRD shows chromatin remodeling activity required for deacetylation. It has been also shown that protein members (HDACs) of the deacetylase complex recruit sequence- specific transcriptional repressors, thereby transcriptional repression by HDACs may not rely solely upon DNA methylation to identify loci to be repressed.

DNA methylation is most commonly associated with transcriptional repression as seen above, but there are at least two cases at imprinted loci H19/Igf2 and Rasgrf1 where DNA methylation of control regions results in transcriptional activation. These control regions are differentially methylated regions (DMRs) that possess enhancer- blocking activity. In an unmethylated state CTCF is able to bind DMRs and thereby blocking the interaction enchancer- promoter of the imprinted genes preventing the transcriptional activation. On the other hand, methylation of DMRs prevents CTCF binding to these domains, allowing the enhancer to come in close proximity with the promoter and to activate transcription of Igf2 and Rasgrf1. The mechanism by which CTCF prevents transcription may involve the binding of SIN3A and associated HDACs, and possibly additional co- repressors.

6. NER factors involved in DNA demethylation besides DNA repair

During the last 5 years, a series of studies demonstrated the function of several NER factors in processes beside DNA repair.

Schmitz at al. showed the necessity of NER factors to recruit on the promoters of rRNA genes in order to facilitate DNA demethylation and the expression of those genes, something that is controlled by the transcriptional factor TAF12 and the Gadd45α (growth arrest and DNA damage inducible protein 45 alpha) (Schmitz K.M. et al., 2009). Le May et al. used an inducible cell differentiation system, and showed the recruitment of several NER factors on the promoters of those induced genes in the absence of genotoxic stress. Moreover, using down-regulated NER factors in a cell culture system, they were able to suggest that NER factors like XPC, XPA, XPF and ERCC1 are essential for the recruitment of Gadd45α on the promoters of activated genes, which is followed by DNA demethylation and histone post-translational modifications (Le May N. et al., 2010). Kamileri et al. working on the *Ercc1*$^{-/-}$ mouse, recently showed the recruitment of ERCC1-XPF with Pol II and the basal transcription factors on promoters of genes associated with postnatal murine growth. Using an inducible cell differentiation assay, the authors were able to show the necessity of ERCC1-XPF in facilitating transcription. Moreover, they observed DNA demethylation upon assembly of ERCC1-XPF on the promoters of growth genes, in association with specific histone marks representing active transcription (Kamileri I et al., 2012). These results are in line with the data presented by Barreto at al. where XPG interacts with Gadd25a and is required to facilitate promoter demethylation during transcription (Barreto G et al., 2007), supporting a similar mechanism where ERCC1-XPF is required to facilitate active demethylation of promoters. Recently, Le May at al. showed that XPG and XPF are essential for establishing CTCF-dependent chromatin looping in the *RARβ2* gene. This process is facilitated by the XPG endonuclease which promotes DNA breaks and DNA demethylation at promoters (Le May N. et al., 2012).

7. Possible link between NER and imprinting

These new findings open new possibilities that may well explain the mechanisms underlying the pathology seen in several NER syndromes. The fact that ERCC1-XPF is involved in DNA demethylation and *Ercc1*$^{-/-}$ mice exhibit developmental defects prompted us to examine

the effects of ERCC1 in genomic imprinting. As it is well known, the molecular mechanism that is responsible for appropriate expression of the imprinted genes which are essential for proper development of an organism is governed by DNA methylation marks.

RESULTS

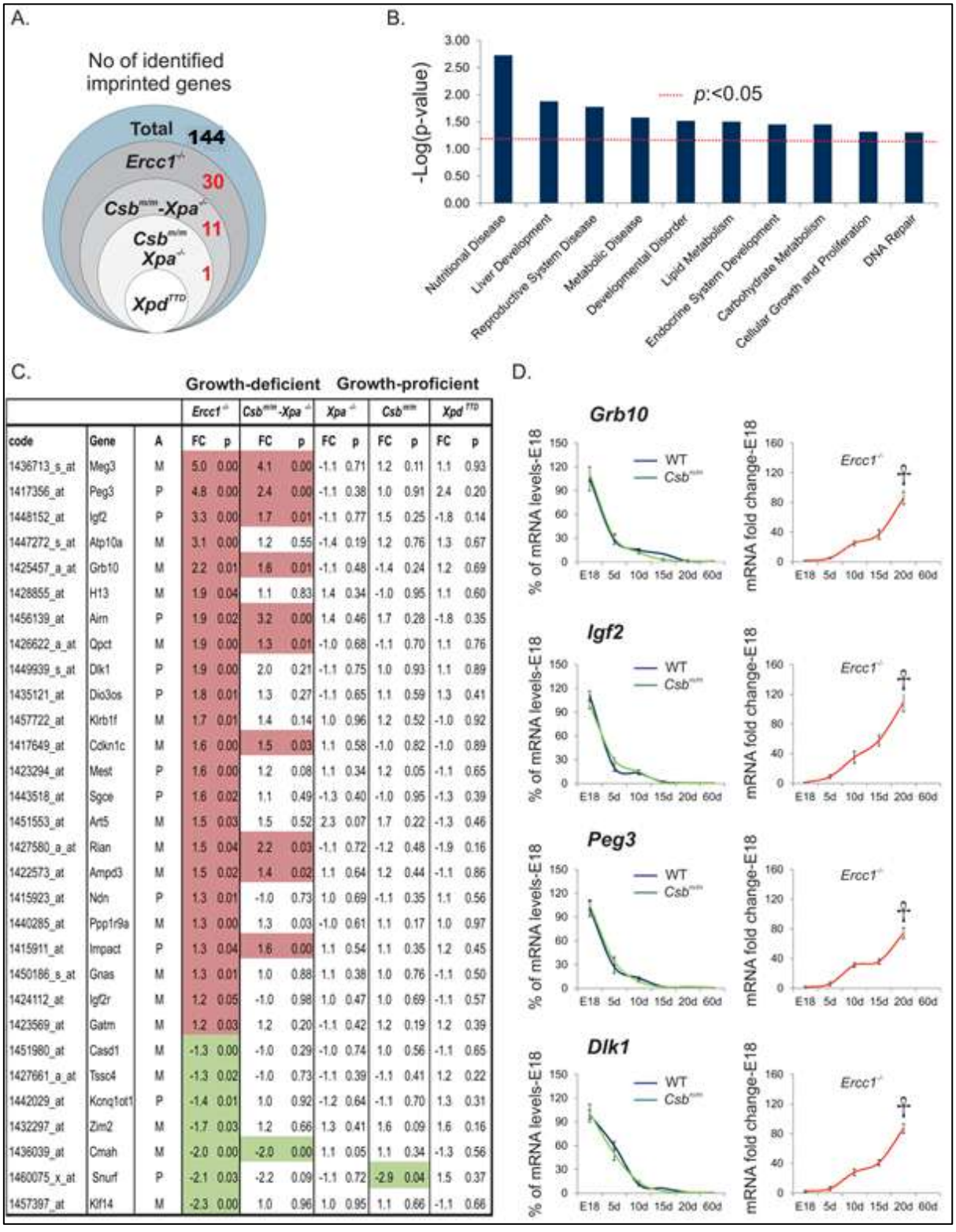
1. Transcriptome analysis reveals aberrant expression of imprinted genes in growth-defective NER mutant animals during development

To examine whether there genomic imprinting is perturbed in *Ercc1*$^{-/-}$ as well as in other NER-deficient mouse mutants (*Csb*$^{m/m}$ - *Xpa*$^{-/-}$, *Csb*$^{m/m}$, *Xpa*$^{-/-}$, and *Xpd*^{TTD}), we looked into available transcriptome data sets derived from 15-day old livers.

Our transcriptome analysis in the livers derived from a series of NER-deficient animals revealed an increase in the expression levels of several imprinted genes as compared to the wild type livers. The greatest effect was seen in *Ercc1*$^{-/-}$ livers, where 30 imprinted genes demonstrated abnormal gene expression levels as compared to wild type livers. This finding can be translated into 20% of the total number of imprinted genes (about 144) in the mouse genome. In *Csb*$^{m/m}$ – *Xpa*$^{-/-}$ double mutant mice we found 11 imprinted genes with abnormal expression levels. Single mutant *Csb*$^{m/m}$ and *Xpa*$^{-/-}$ mice show only 1 imprinted gene with abnormal expression levels, whereas *Xpd*^{TTD} mice showed none of the aforementioned gene expression changes (Figure 1A). Double and single mutant mice for the imprinted genes *Csb* and *Xpa*, affect less than 8% of the total number of imprinted genes in the mouse genome.

Interestingly, although these proteins participate in the same pathway (NER), the number of the affected imprinted genes varies dramatically between the different animals carrying a different NER defect. In particular, the *Ercc1*$^{-/-}$ liver exhibit nearly three times more affected imprinted genes than the double mutant for the NER factor genes *Csb* and *Xpa*, and almost 20 times more affected imprinted genes than the single mutants for the genes *Csb* and *Xpa*. This observation indicates a functional role for Ercc1 during genomic imprinting in addition to NER.

Next, we explored the biological processes that underlie the imprinted genes that show aberrant gene expression changes in *Ercc1* livers. To do this, we first grouped all genes according to their known or predicted biological function into gene ontology (GO) categories. Next, we asked which GO terms are significantly over-represented among the significantly differentially expressed genes in *Ercc1*$^{-/-}$ mice (see Methods). This approach revealed ten



			Ercc1-/-		Csbm/m-Xpa-/-		Xpa-/-		Csbm/m		XpdTTD	
code	Gene	A	FC	p	FC	p	FC	p	FC	p	FC	p
1436713_s_at	Meg3	M	5.0	0.00	4.1	0.00	-1.1	0.71	1.2	0.11	1.1	0.93
1417356_at	Peg3	P	4.8	0.00	2.4	0.00	-1.1	0.38	1.0	0.91	2.4	0.20
1448152_at	Igf2	P	3.3	0.00	1.7	0.01	-1.1	0.77	1.5	0.25	-1.8	0.14
1447272_s_at	Atp10a	M	3.1	0.00	1.2	0.55	-1.4	0.19	1.2	0.76	1.3	0.67
1425457_a_at	Grb10	M	2.2	0.01	1.6	0.01	-1.1	0.48	-1.4	0.24	1.2	0.69
1428855_at	H13	M	1.9	0.04	1.1	0.83	1.4	0.34	-1.0	0.95	1.1	0.60
1456139_at	Airn	P	1.9	0.02	3.2	0.00	1.4	0.46	1.7	0.28	-1.8	0.35
1426622_a_at	Qpct	M	1.9	0.00	1.3	0.01	-1.0	0.68	-1.1	0.70	1.1	0.76
1449939_s_at	Dlk1	P	1.9	0.00	2.0	0.21	-1.1	0.75	1.0	0.93	1.1	0.89
1435121_at	Dio3os	P	1.8	0.01	1.3	0.27	-1.1	0.65	1.1	0.59	1.3	0.41
1457722_at	Klrb1f	M	1.7	0.01	1.4	0.14	1.0	0.96	1.2	0.52	-1.0	0.92
1417649_at	Cdkn1c	M	1.6	0.00	1.5	0.03	1.1	0.58	-1.0	0.82	-1.0	0.89
1423294_at	Mest	P	1.6	0.00	1.2	0.08	1.1	0.34	1.2	0.05	-1.1	0.65
1443518_at	Sgce	P	1.6	0.02	1.1	0.49	-1.3	0.40	-1.0	0.95	-1.3	0.39
1451553_at	Art5	M	1.5	0.03	1.5	0.52	2.3	0.07	1.7	0.22	-1.3	0.46
1427580_a_at	Rian	M	1.5	0.04	2.2	0.03	-1.1	0.72	-1.2	0.48	-1.9	0.16
1422573_at	Ampd3	M	1.5	0.02	1.4	0.02	1.1	0.64	1.2	0.44	-1.1	0.86
1415923_at	Ndn	P	1.3	0.01	-1.0	0.73	1.0	0.69	-1.1	0.35	1.1	0.56
1440285_at	Ppp1r9a	M	1.3	0.00	1.3	0.03	-1.0	0.61	1.1	0.17	1.0	0.97
1415911_at	Impact	P	1.3	0.04	1.6	0.00	1.1	0.54	1.1	0.35	1.2	0.45
1450186_s_at	Gnas	M	1.3	0.01	1.0	0.88	1.1	0.38	1.0	0.76	-1.1	0.50
1424112_at	Igf2r	M	1.2	0.05	-1.0	0.98	1.0	0.47	1.0	0.69	-1.1	0.57
1423569_at	Gatm	M	1.2	0.03	1.2	0.20	-1.1	0.42	1.2	0.19	1.2	0.39
1451980_at	Casd1	M	-1.3	0.00	-1.0	0.29	-1.0	0.74	1.0	0.56	-1.1	0.65
1427661_a_at	Tssc4	M	-1.3	0.02	-1.0	0.73	-1.1	0.39	-1.1	0.41	1.2	0.22
1442029_at	Kcnq1ot1	P	-1.4	0.01	1.0	0.92	-1.2	0.64	-1.1	0.70	1.3	0.31
1432297_at	Zim2	M	-1.7	0.03	1.2	0.66	1.3	0.41	1.6	0.09	1.6	0.16
1436039_at	Cmah	M	-2.0	0.00	-2.0	0.00	1.1	0.05	1.1	0.34	-1.3	0.56
1460075_x_at	Snurf	P	-2.1	0.03	-2.2	0.09	-1.1	0.72	-2.9	0.04	1.5	0.37
1457397_at	Klf14	M	-2.3	0.00	1.0	0.96	1.0	0.95	1.1	0.66	-1.1	0.66

Figure 1. A) Summary of the impact of affected imprinted genes in five NER mutants (Ercc1$^{-/-}$, Csb$^{m/m}$ – Xpa$^{-/-}$, Csb$^{m/m}$, Xpa$^{-/-}$, and Xpd^{TTD}). B) Gene ontology (GO) terms that are over-represented among the significantly differentially expressed imprinted genes in *Ercc1*$^{-/-}$ livers. C) Representation of the 30 perturbed imprinted genes in *Ercc1*$^{-/-}$ livers and comparison to different NER mutants. In red and green are marked the genes that are

significantly upregulated and downregulated respectively, compared to the wild type. FC: fold change; P: p-value. D) Relative Grb10, Igf2, Peg3, and Dlk1 mRNA levels during postnatal Ercc1$^{-/-}$, Csb$^{m/m}$ and wt liver development (n ≥ 4; E, embryonic; d, days). Wild type and Csb$^{m/m}$ mRNA levels are expressed as percentage over E18 mRNA levels, whereas Ercc1$^{-/-}$ levels are expressed as fold enrichment over E18 mRNA levels.

biological processes altered in *Ercc1*$^{-/-}$ livers (Figure 1B). These processes were ranked by their relative enrichment score (see Methods) and included: (i) nutritional pathways, (ii) pathways involved in hepatic development and (iii) reproduction, (iv) carbohydrate (v) lipid, and (vi) endocrine metabolism as well biological processes related to cellular growth, proliferation and (vii) DNA repair. Together, these findings accurately reflect the growth defect seen in *Ercc1*$^{-/-}$ animals suggesting that a causal association between the aberrant regulation of the observed pathways and the dramatic pathology seen in *Ercc1*$^{-/-}$ progeroid animals.

Figure 1C shows the perturbed imprinted genes in the different NER factor mutants. In *Ercc1*$^{-/-}$ livers 23 out of the 30 imprinted genes which show abnormal expression levels are upregulated (depicted in red), whereas the expression levels of the other 7 imprinted genes are downregulated (depicted in green). As shown in Figure 1C, the growth-deficient animals (*Ercc1*$^{-/-}$ and *Csb*$^{m/m}$ – *Xpa*$^{-/-}$), showed perturbed imprinted expression, in comparison to the remaining three NER mutants who are growth-proficient. Moreover, it should be mentioned that there is no bias towards the paternal or the maternal perturbed imprinted genes in the two growth-deficient mice.

2. Relative mRNA expression levels of *Igf2*, *Grb10*, *Peg3*, *Dlk1* in *Ercc1*$^{-/-}$ livers during development

We next asked whether the relative mRNA expression levels of the imprinted genes in *Ercc1*$^{-/-}$ livers are affected during development.

To do this, we focused our work on 4 well characterized imprinted genes, such as the *Igf2*, *Grb10*, *Peg3*, and *Dlk1* whose gene expression is upregulated in *Ercc1*$^{-/-}$ livers. We compared the expression levels of those genes to the wild type livers, by quantitative PCR (qPCR) during postnatal development (E18, 5d, 10d, 15d, 20, and 60d).

As shown in Figure 1D, in wild type livers the expression levels of all four imprinted genes drops dramatically over time. In contrast, in *Ercc1*$^{-/-}$ livers, the mRNA levels of imprinted genes increases postnatally with advancing age. In *Csb*$^{m/m}$ livers, however, the mRNA levels of imprinted genes showed a similar gene expression profile with that previously seen in wild type livers. These findings further confirm the microarray data.

Together, our findings suggest that the perturbed expression of the imprinted genes in *Ercc1*$^{-/-}$ livers occurs during postnatal development. It remains to be seen whether the up-regulation of imprinted genes is due to the overexpression of the already expressed alleles, or it reflects the biallelic expression of these genes, thereby indicating loss of imprinting.

3. Promoter occupancies of RNA Pol II and the basal transcription factors on *Igf2*, *Grb10*, *Dlk1* and *Peg3* genes in *Ercc1*$^{-/-}$ livers

We next asked whether the basal transcription factors required for proper transcription are recruited on the promoters of imprinted genes in 15d-old *Ercc1*$^{-/-}$ livers as compared to the wild type animals.

To do this, we examined the recruitment of the RNA polymerase II and components of the basal transcription machinery (TFIIH, XPD, and TAF5) on the promoters of *Igf2*, *Grb10*, *Dlk1*, and *Peg3* (Figure 2A). We used chromatin immuneprecipitation (ChIP) assays with antibodies against RNA Pol II, TFIIH, XPD (XPD is a subunit of TFIIH), and TAF5 followed by qPCR specific for the promoters of the four imprinted genes. As a negative control for the ChIP, we used the antibody α-Fras1 against the FRAS1 protein, a membrane protein that is not supposed to be found on promoters.

Unlike the wt livers, RNA polymerase II, and TFIIH, XPD, and TAF5 were recruited on the promoters of *Igf2*, *Grb10*, *Dlk1*, and *Peg3* in *Ercc1*$^{-/-}$ livers. Although the occupancy of the promoters by the transcriptional factors and the Pol II correlates with the previously shown up-regulation of imprinted genes during P15 (see Figure 1D), it is impossible to determine whether this is due to loss of imprinting or it represents an overexpression of the already expressed allele.

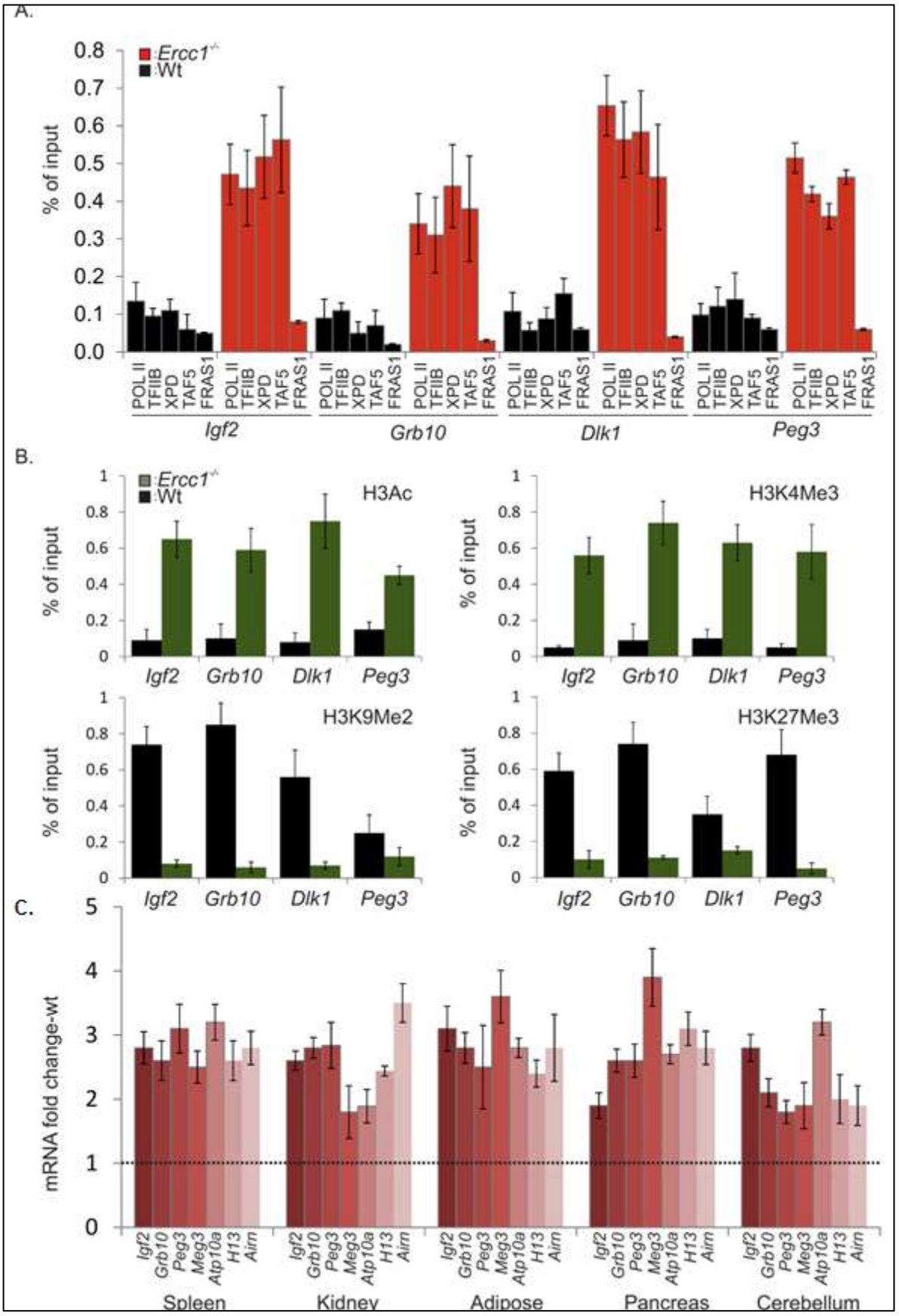


Figure 2. A) ChIP assays with antibodies against the POL II, TFIIB, XPD, TAF5, and FRAS1 in P15 *Ercc1*$^{-/-}$ livers. The data from the qPCR reactions with primers amplifying the

promoter regions of the tested imprinted genes *Igf2*, *Grb10*, *DLK1*, and *Peg3* in *Ercc1*$^{-/-}$ livers ($n \geq 4$) were normalized to input and expressed as percentage of input over those obtained from wt controls. B) ChIP signals for activating (H3Ac, H3K4Me3) and repressive (H3K9Me2, H3K27Me3) histone modifications at the indicated promoter regions of the imprinted genes in P15 *Ercc1*$^{-/-}$ livers ($n \geq 4$). The data from the q-PCR reactions with primers amplifying the promoter regions of the tested imprinted genes *Igf2*, *Grb10*, *DLK1*, and *Peg3* in *Ercc1*$^{-/-}$ livers ($n \geq 4$) were normalized to input and expressed as percentage of input over those obtained from wt controls. C) Q-PCR evaluation of mRNA levels of imprinted genes in Spleen, Kidney, Adipose, Pancreas, and Cerebellum of 15d old *Ercc1*$^{-/-}$ pups. For each gene, expression levels in the *Ercc1*$^{-/-}$ tissues are plotted relative to those of age-matched controls (black dotted line). Error bars indicate SEM between replicates ($n \geq 4$).

4. Histone PTMs in *Igf2, Grb10, Dlk1* and *Peg3* promoter regions in *Ercc1*$^{-/-}$ livers

Next, we looked into the chromatin status on the promoters of the imprinted genes. Post translational modifications of histones such as H3Ac and H3K4Me3 are hallmarks of active transcription, and those like H3K9Me2 and H3K27Me3 are found in closed and silenced chromatin.

To this end, we immunoprecipitated the chromatin with specific antibodies against the above mentioned PTMs for each residue, followed by qPCR using primers specific for the promoters of the four imprinted genes (Figure 2B).

We observed higher levels of active transcription histone marks, and subsequently lower levels of closed and silenced chromatin on the promoters in 15d *Ercc1*$^{-/-}$ livers compared to the wild type livers. This finding indicates that in *Ercc1*$^{-/-}$ livers the chromatin configuration surrounding the promoter regions of interest is more relaxed favouring transcription.

5. Aberrant expression of imprinted genes in *Ercc1*$^{-/-}$ organs

To explore whether the perturbed expression of the imprinted genes observed in 15d *Ercc1*$^{-/-}$ livers (see Figures 1C and 1D) is systemic in *Ercc1*$^{-/-}$ mice, we analyzed the relative expression levels of several imprinted genes in different organs of 15d *Ercc1*$^{-/-}$ mice (Figure 2C).

Quantitative PCR was performed in spleen, kidney, adipose, pancreas, and cerebellum using primers specific for the imprinted genes *Igf2, Grb10, Peg3, Meg3, Atp10a, H13,* and *Airn*.

All the imprinted genes tested in the various organs of 15d *Ercc1*$^{-/-}$ show increased mRNA levels compared to the wild type organs. Thus, the perturbed expression of the imprinted genes affects many organs and is not only restricted to the *Ercc1*$^{-/-}$ livers.

6. Relative mRNA expression levels of DDR and imprinted genes in UV-irradiated MEFs and MEFs exposed to H_2O_2

As NER proteins are typically associated with DNA repair, we asked whether the perturbed expression of the imprinted genes seen in *Ercc1*$^{-/-}$ livers is a response to irreparable DNA damage.

To do this, we exposed wild type mouse embryonic fibroblast (MEF) cells which are known to express imprinted genes, to two different sources of DNA damage, UV irradiation (Figure 3A) as well as hydrogen peroxide [H_2O_2] (Figure 3B). Following treatment, we measured the mRNA expression levels of known genes that are responsive to UV light, and hydrogen peroxide respectively by q-PCR.

Unlike for the UV-responsive genes, MEFs exposed to 0.6 J/m^2 UV light show no upregulation in the expression levels of the imprinted genes tested. This was more profound when MEFs were exposed to 4 J/m^2, where a 2- to 4-fold upregulation of the UV-responsive genes was observed; importantly, the imprinted genes showed no response to the increased dose UV light. Unlike the imprinted genes, MEFs also exposed to 50 mM hydrogen peroxide showed an upregulation of the H_2O_2-responsive genes.

Taken together, our findings exclude the possibility that the perturbed expression of the imprinted genes observed in *Ercc1*$^{-/-}$ livers and in other organs is a response to DNA damage. At the same time it strengths the argument that Ercc1 and possible other NER factors have novel functions beyond the pathway of NER. Alternatively, genomic imprinting might only be fully functional in vivo; MEFs may therefore be an inadequate system to explore the effects of DNA damage on genomic imprinting during postnatal development or with advancing age.

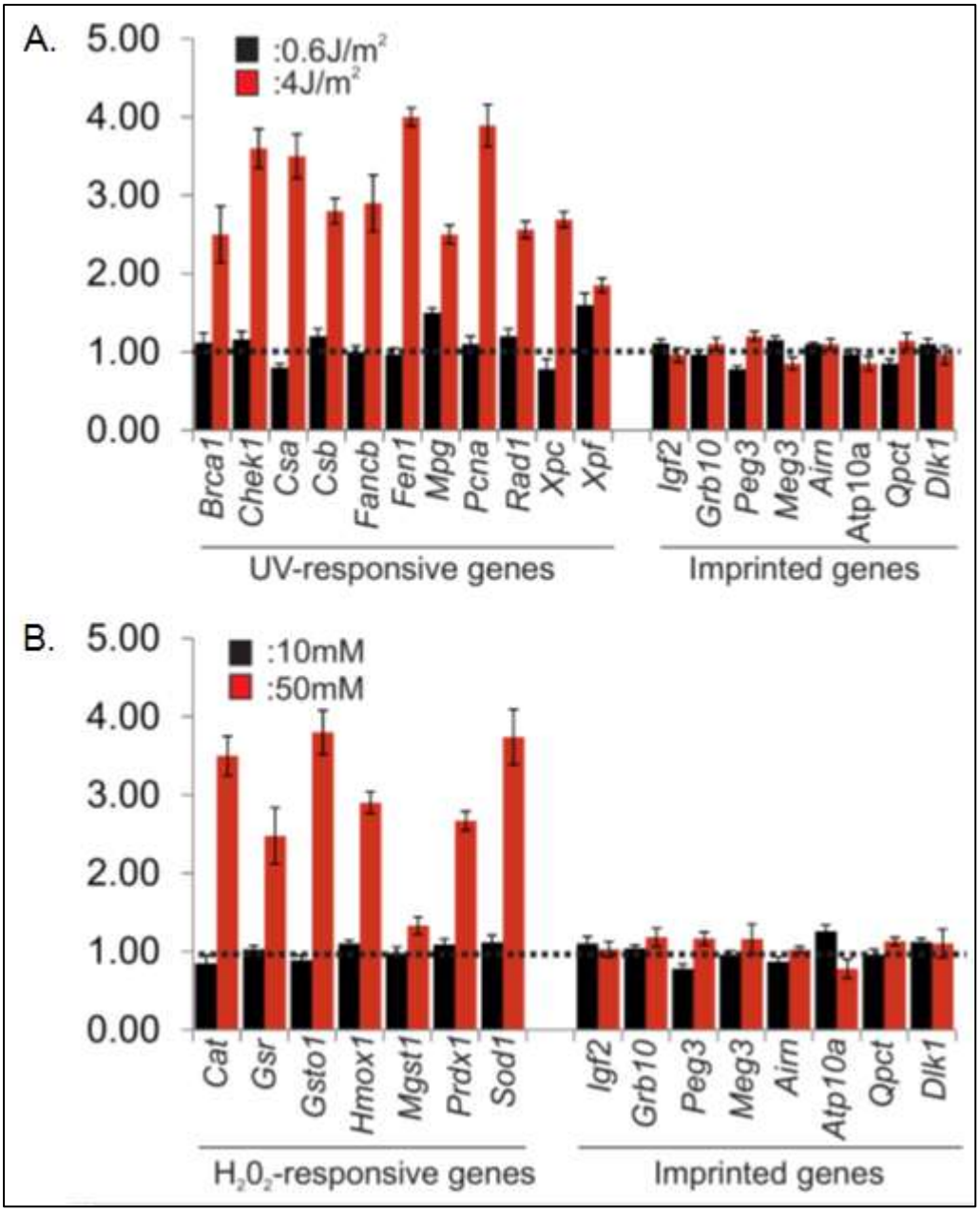


Figure 3. Wild type MEFs exposed to two different sources of DNA damage, UV light (A) and hydrogen peroxide (B). Thereafter, expression levels of two groups of genes have been evaluated by Q-PCR. The first group comprises genes that are responsive either to UV light or hydrogen peroxide, and the second group consists of imprinted genes. Black colour stands for low dose of either UV light or hydrogen peroxide where no response to DNA damage is triggered by the cells, and red stands for high dose of the DNA damaging agents where responsive genes are activated.

DISCUSSION

Dissecting the dual role of NER in DNA repair and transcription, particularly in an intact organism, has always been challenging. Indeed, there is still much to be done before we are able to fully understand how NER functions in development and disease. Using more sophisticated mouse models (e.g., tissue-specific or knockin animals and high-throughput approaches) might allow us to gain further insights into disease origin and progression or to develop rationalized intervention strategies aimed at counteracting the numerous clinical complications associated with NER syndromes, including progeria and cancer. A connection between the presence of irreparable DNA lesions in the mammalian genome (due to the DNA repair defect) and loss of genomic imprinting remains currently unknown. Since several mutant mice carrying defects in NER do not show a similar deregulation in gene expression changes to that observed in *Ercc1*$^{-/-}$ mice, our findings suggest that DNA damage per se may not be causal to the observed loss of imprinting in the aforementioned genes. We and others have previously shown that distinct NER proteins have functions in transcription or chromatin-based regulation of gene expression that go well beyond DNA repair. In our lab, recent work revealed a functional link between the ERCC1-XPF complex and the basal transcription machinery at promoters: ERCC1-XPF interacts with TAFs and TBP and assembles together with RNAPII and the basal transcription factors at the promoters of hepatic genes during postnatal development (Kamileri at al., 2012). Interestingly, in this study, targeting ERCC1-XPF and Gadd45α at promoters greatly facilitated promoter DNA demethylation and the appearance of histone posttranslational modifications associated with active transcription. This was confirmed by disruption of ERCC1 in *Ercc1*$^{-/-}$ livers, which led to promoter DNA hypermethylation, heterochromatic histone marks, and reduced mRNA levels of growth genes. Even so, there is no evidence for a direct function of ERCC1-XPF in repair-mediated DNA demethylation. Instead, the recent findings make it more likely that the NER complex indirectly facilitates promoter DNA demethylation by maintaining a euchromatin environment associated with active transcription. In turn, the presence of transcription-blocking DNA lesions could further aggravate the transcriptional defect in *Ercc1*$^{-/-}$ mice, or increased DNA damage levels might render NER factors inaccessible for transcription initiation. Taken together, the defect in DNA repair may well explain the UV sensitivity of XP, CS, and TTD patients and corresponding animal models. Likewise, the

accumulation of irreparable DNA lesions over time could provide a rational basis for the increased cancer predisposition seen in patients with Xeroderma Pigmentosum or the gradual appearance of progeroid features seen in Cockayne Syndrome, Trichothiodystrophy, and XFE. However, the developmental defects in NER (and the remarkable heterogeneity observed therein), including the observed loss of genomic imprinting are better explained by the NER defect in processes other than DNA repair, including transcription. Their severity would mirror the complexity of gene expression programs and the individual tissue requirements (in which the NER defect is found) during mammalian development.

MATERIAL AND METHODS

1. Microarray hybridizations and quantitative real-time PCR analysis

Standard procedures were used to obtain total RNA (Qiagen) from the liver of wt, *Ercc1*$^{-/-}$ and *Taf10*$^{-/-}$ animals (n=4) at the indicated timepoints. For genome-wide expression analysis of *Taf10*$^{-/-}$ or *XpdTTD* livers, synthesis of double stranded cDNA and biotin labelled cRNA was performed according to the instructions of the manufacturer (Affymetrix, USA). Fragmented cRNA preparations were hybridized to full mouse genome oligonucleotide arrays (430 V2.0; Affymetrix, USA). Initial data extraction and normalization within each array was performed by means of the GCOS software (Affymetrix). Additional data were collected from cited sources: 2 week-old DNA repairdeficient (*i.e.*, *Csb*$^{m/m}$*;Xpa*$^{-/-}$, *Ercc1*$^{-/-}$) and age-matched littermate control mice (*Csb*$^{m/m}$, *Xpa*$^{-/-}$ and corresponding wt) (1, 4). Microarrays complied with the Minimum Information for Microarray Experiments (MIAME) and are available at ArrayExpress (EMEXP-1503, E-MEXP-835 and E-MEXP-3442). All qPCR reactions were done in triplicates using Biorad MyIQ, SYBR GreenI (Sigma) and Platinum Taq polymerase (Invitrogen). Generation of specific PCR products was confirmed by melting curve analysis, gel electrophoresis and sequencing. Primer pairs were tested with a logarithmic dilution cDNA to generate a linear standard curve (crossing point (CP) plotted versus log of template concentration), which was used to calculate the primer pair efficiency ($E = 10(-1/\text{slope})$). For data analysis, the second derivative maximum method was applied, and induction of target cDNA was calculated as previously described (5): $(E_{target}^{\Delta CP(cDNA_{untreated}-cDNA_{treated})_{target}}) / (E_{control}^{\Delta CP(cDNA_{untreated}-cDNA_{treated})_{control}})$. Primer sequences for the genes tested in qPCR are available upon request.

2. Gene ontology (GO) classification and overrepresentation of biological themes

All significant gene entries were subjected to GO classification (http://www.geneontology.org). Significant overrepresentation of GO-classified biological

processes was determined by comparing the number of genes in a given biological process that were significantly differentially expressed in a particular mouse strain to the total number of the genes relevant to that biological process printed on the array (Fisher exact test, $p \leq 0.01$ False discovery rate (FDR) ≤ 0.1) using the publicly accessible software Ease and/or DAVID (http://david.abcc.ncifcrf.gov/summary.jsp). Due to the redundant nature of GO annotations, we employed kappa statistics to measure the degree of the common genes between two annotations and heuristic clustering to classify the groups of similar annotations according to kappa values (http://david.abcc.ncifcrf.gov/summary.jsp). Significant overrepresentation of pathways and gene networks was determined by DAVID (http://david.abcc.ncifcrf.gov/summary.jsp; through BBID, BIOCARTA and KEGG annotations) as well as by means of the ingenuity pathway analysis software (www.ingenuity.com).

3. Chromatin immunoprecipitation

For ChIP assays, tissues were minced in PBS and were crosslinked at R.T. for 10 min with 1% formaldehyde while cells where homogenized in glass dounce. The cross-linking in the cells was achieved in standard medium with the same conditions without homogenization. The cross-linking was stopped by addition of 0.125M glycine. Samples were centrifuged for 5min at 4C at 2000rpm. Pellets were resusdended in 10ml 1xPBS/PMSF buffer and centrifuged for 5min at 4C at 2000rpm. Pellets were resuspended in 10ml B Buffer (0.25% TritonX-100, 10mM EDTA, 0.5mM EGTA, 20mM HEPES, aprotinin/PMSF) rotated for 10min at 4C and centrifuged for 5min at 4C at 2000rpm. Pellets where were resuspended in 10ml C Buffer (0.15M NaCl, 1mM EDTA, 0.5mM EGTA, 20mM HEPES, aprotinin/PMSF) rotated for 10min at 4C and centrifuged for 5min at 4oC at 2000rpm. Pellet which contains the nuclei were resuspended in Sonicatinon Buffer (50mM Hepes pH 7.9, 140mM NaCl, 1mM EDTA, 1% Triton X-100, 0.1% Na deoxycolate, 0,1% SDS, 0.5mM PMSF, protease inhibitor cocktail (Roche)). Chromatin was prepared and sonicated on ice 6 x 15sec using an Ultrasonic homogenizer. Samples were immunoprecipitated with antibodies and protein G-Agarose beads (Millipore, USA) at 4C overnight and washed sequentially 2 times with Sonication Buffer, Wash Buffer A (50mM Hepes pH 7.9, 500mM NaCl, 1mM EDTA, 1% Triton X-100, 0.1% Na deoxycolate, 0,1% SDS, 0.5mM PMSF, protease inhibitor cocktail (Roche)) , Wash Buffer B (50mM Hepes pH 7.9, 1mM EDTA, 250mM LiCl, 0.5% Na

deoxycolate, 0.5% NP-40, 0.5mM PMSF, protease inhibitor cocktail (Roche)), 1xTE. The complexes were eluted twice in 150µl Elution Buffer (50mM Tris pH 8.0, 1mM EDTA, 1% SDS, 50mM NaHCO3) and the crosslinking was heat reversed 5 hours in 65C. Samples were incubated 1 hour with RNAse A (Invitrogen) at 37C and 2 hours with EDTA and Proteinase K at 42C. DNA was extracted two times with phenol/chlorophorm/isoamylalcohol (Fluka) and once with chlorophorm (Merck) by centrifuging 14.000rpm for 10min and supernatant was collected in a new tube.

4. RNA extraction

Total RNA was isolated from livers with Trizol (Invitrogen). Livers were mechanically homogenized in 1ml Trizol, transferred in ependorf tubes and incubated in Room Temperature (RT) for 5min. 200µl Chlorophorm (Merck) were added and centrifuged at 12.000g for 15min. Supernatants were transferred in a new tube, 500µl isopropanol (Fluka) were added and samples were incubated in RT for 10min. Samples were centrifuged at 12.000g for 10min and pellets were washed in 1ml 70% Ethanol. Pellets were air died and RNA was diluted in water.

5. cDNA synthesis

1µg of total RNA were used in two steps cDNA synthesis. First, RNA was incubated with OligodT (Invitrogen) at 72C for 10min and then 5x SuperScript Buffer (Invitrogen), DTT (Invitrogen), RNAout (Invitrogen) and Superscript (Invitrogene).

6. Quantitive real time PCR

For the quantitive real time PCRs 1µl of cDNA was used in 25 µl final volume:

10x Platinum Taq Buffer (Invitrogen):	25µl
50mM MgCl2 (Invitrogen):	1.25µl
10mM dNTPs (Invitrogen):	0.4 µl
Pr1 (1.25pm/µl):	2 µl

Pr2 (1.25pm/μl): 2 μl

Syber green (1/2000) (Invitrogen): 0.75 μl

Platinum Taq Polymerases (Invitrogen): 0.1 μl

Conditions for the PCR reaction:

Step1: 94C for 5min

Step2: 94C for 15sec

Step3: 60C for 25sec

Step4: 72C for 25sec

Step5: plate read

Step6: 78C for 1sec

Step7: plate read

Step8: Go to step 2 for 38 times

Step9: Incubate 72C for 2sec

7. Isolation of Mouse Embryonic Fibroblasts

A pregnant female was sacrificed at the day 13.5 of gestation. The dissection was performed in sterile conditions in hood. All the needed materials were sterile at UV before used. The embryos were dissected out of the extraembryonic membranes and washed in 1xPBS. The soft organs and the viscera (e.g. liver, brain, gut etc.) were dissected away. The remaining carcass were cut into small pieces with a sterile scissor and throw into 15ml falcon tubes containing 6ml 1x trypsin. From wt females, 3 embryos were put in one falcon, from het females, each embryo was put in one falcon. The falcons were placed in 37C for 30min. The samples were pipette up and down until homogenize with sterile Pasteur pipettes and then centrifuged for 5min at 1,500rpm at Room Temperature. The pellet was resuspended in MEF medium and placed into flasks. The cells were incubated at 37C, 5% CO_2.

LITERATURE

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