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Mini review

Dynamics of mammalian NER proteins

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ABSTRACT

Despite detailed knowledge on the genetic network and biochemical properties of most of the nucleotide excision repair (NER) proteins, cell biological analysis has only recently made it possible to investigate the temporal and spatial organization of NER. In contrast to several other DNA damage response mechanisms that occur in specific subnuclear structures, NER is not confined to nuclear foci, which has severely hampered the analysis of its arrangement in time and space. In this review the recently developed tools to study the dynamic molecular transactions between the NER factors and the chromatin template are summarized. First, different procedures to inflict DNA damage in a part of the cell nucleus are discussed. In addition, technologies to measure protein dynamics of NER factors tagged with the green fluorescent protein (GFP) will be reviewed. Most of the dynamic parameters of GFP-tagged NER factors are deduced from different variants of 'fluorescence recovery after photobleaching' (FRAP) experiments and FRAP analysis procedures will be briefly evaluated. The combination of local damage induction, genetic tagging of repair factors with GFP and microscopy innovations have provided the basis for the determination of NER kinetics within living mammalian cells. These new cell biological approaches have disclosed a highly dynamic arrangement of NER factors that assemble in an orderly fashion on damaged DNA. The spatio-temporal analysis tools developed for the study of NER and the kinetic model derived from these studies can serve as a paradigm for the understanding of other chromatin-associated processes.

Contents

1. Introduction

Several decades of genetic and biochemical research have culminated into a comprehensive molecular model that describes the subsequent steps of the nucleotide excision repair (NER) mechanism in eukaryotes [1]. Phenotypic characterization and epitasis analysis of DNA damage hyper-sensitive mutants from different model organisms, mainly *Saccharomyces cerevisiae* together with laboratory induced NER-deficient Chinese Hamster ovary cells and cells derived from hereditary NER-deficient patients (XP, CS or TTD) have provided the genetic framework to identify, clone and

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characterize eukaryotic NER genes. Homology-based analysis and peptide-domain identification of predicted open reading frames encoded by the isolated NER genes have further provided the groundwork for a better understanding of the NER process. The largest increment towards insight into the NER mechanism was however made by the development of an *in vitro* NER assay [2–4]. Recent, structural analysis of some of the key NER factors were very important to understand how these proteins interact with the substrate (damaged DNA) [5,6]. Generation of NER-mutant mice, either full knock-outs or patient mimicking knock-in mice have provided valuable insight into the etiology of the pathology associated to specific NER-deficiencies [7]. Finally, gene expression analyses of some NER-deficient mouse-models were instrumental towards unraveling (adaptive) responses of repair defects at the organismal level [8,9].

Only about a decade ago a new biological research area emerged that allowed the analysis of protein dynamics in living cells, using tagging of proteins with the green fluorescent protein (GFP) and quantitative live cell microscopy and spectroscopy. The analysis of GFP-tagged NER factors was at the start of these novel developments. Measuring of NER factors in living cultured cells [10] largely contributed to the development of the live cell microscopy analysis-tools [11–13]. The prior conditions for such a research endeavor, i.e. genetic basis and detailed biochemical properties were all available within the NER field. Detailed knowledge on several parameters made the NER process ideally suited to serve as a paradigm to study chromatin-associated processes in living cells.

2. Spatial organization of NER in mammalian cells

The above mentioned multi-disciplinary approaches were instrumental to obtain a detailed view into the complex molecular machinery of NER. The current consensus for the NER mechanism suggests that multiple proteins assemble in a hierarchical sequential manner on the damaged site and cooperate in an intricate manner to: – recognize DNA helix distortions, – select or verify whether disturbed local base pairing is caused by a proper type of DNA lesion to be repaired by NER, – recruit and properly orient the structure specific endonucleases, which make ssDNA incisions at both sites surrounding the lesions, – and finally ssDNA gap filling synthesis and ligation. Obviously, a highly spatial and temporal orchestration of the many molecular interactions is required to allow efficient and faithful excision and final restoration of damaged sites. Although the genetic and biochemical studies and the availability of an *in vitro* reconstituted NER system provided a wealth of information on the molecular intricacies and the distinct steps within the NER process, knowledge on how these factors find lesions and assemble within the complexity of a mammalian cell nucleus was lacking.

Only, until recently, cellular biological aspects such as the dynamics and spatial organization of mammalian NER proteins have escaped scrutiny, mainly due to the lack of proper methods to visualize NER within mammalian cell nuclei. On the other hand, analysis of the spatial organization of proteins implicated in another repair process; DNA double-strand break (DSB) repair was greatly stimulated by the discovery of the DNA damage induced phosphorylation of the H2A-variant; H2AX by the ATM and ATR kinases [14]. Upon DNA damage induction phosphorylated H2AX (γH2AX) was found restricted to microscopically discernable small nuclear foci; IRIF (for ionizing-radiation induced foci). It turned out that this γH2AX provided a platform for the assembly of a large group of DNA damage response (DDR) factors, including proteins directly involved in the repair of DSB and down-stream signaling factors [15].

Particularly the absence of such microscopically discernable sub-nuclear structures in which NER factors assemble after DNA damage has made it difficult to analyze NER at the cell biological level. Remarkably however, UV-irradiation that produces predominantly the DNA-photoproducts cyclo-butane pyrimidine dimers (CPD) and pyrimidine (6-4) pyrimidone photoproducts (6-4PP) that are known to be processed by NER also induces γH2AX, although UV doesn't directly cause DSBs [16,17]. In contrast to DSBs, UV-irradiation causes a homogeneously distributed γH2AX [17], with the exception of cells in S-phase where also γH2AX foci were formed. These foci in S-phase are likely caused by a secondary effect of UV-lesions, as these will disturb replicative DNA synthesis which may cause fork collapse that leads to DSBs. Surprisingly, H2AX phosphorylation upon UV in non-S-phase cells appeared to depend on active NER and is thought to be triggered by single-strand DNA repair intermediates [18] that activate ATR-dependent but replicative-stress independent signaling [16]. Although the actual chromatin mark (γH2AX) is identical between DSB-induced DDR and UV-induced DDR, in the latter case this histone modification is a consequence of active NER rather than being involved in repair factor assembly as it is the case of DSBs. The notion that γH2AX formation occurs in response to NER and that NER is proficient in H2AX-deficient cells, argues that this histone modification mainly functions beyond actual repair and was suggested to play a role in UV-induced checkpoint activation. Recent findings further provide evidence for a model in which NER-intermediates trigger checkpoint activation [19,20].

High resolution imaging of NER factor distribution in response to UV-light using electron microscopy with immuno-gold labeled antibodies towards XPA and XPC revealed that these factors were enriched in the transcriptionally active and relative open perichromatin regions [21]. Although XPC, but not XPA, was also found moderately enriched in response to UV-irradiation within condensed chromatin domains, a model was suggested in which NER complex formation preferentially occurred in a more open chromatin environment. Alternatively, these observations can also be explained by a model in which the damage sensor XPC detects lesions even within dense chromatin structures and initiated a, thus far unknown, translocation or unfolding of NER complexes to perichromatin regions. This latter possibility is further supported by the notion that UV-damaged chromatin domains undergo surface expansion [21]. Although, this analysis provided novel insight into the spatial distribution of NER and argues for a NER-induced chromatin modification, these studies will not reveal the dynamic molecular transactions within active NER.

3. Local UV-irradiation: a versatile tool to study NER *in situ*

Whereas EM is obviously superior to any light-microscopic procedure in terms of resolution, the required harsh fixation conditions and difficulties of anti-body penetration into tightly fixed material limits the application of EM to visualize NER. EM studies were further hampered by tedious imaging procedures to localize DNA damage and NER factors in globally irradiated cell nuclei. Cell biological NER analysis, using classical immuno-histochemistry and light-microscopy was greatly boosted by the development of procedures to locally inflict DNA damage in a part of the nucleus of living cultured cells. Almost simultaneously, two groups independently developed the same procedure to introduce UV-induced DNA damage in a part of the nucleus *in situ*. The beauty of this procedure lies in its simplicity. Cultured adherent cells were covered with a microporous filter and subsequently irradiated with UV-C light (Fig. 1A and B) [22,23]. The membrane functions as an irradiation mask by shielding the cells from UV-light except at the positions of the (micro-meter sized) pores in the filter. Partial shielding and subse-

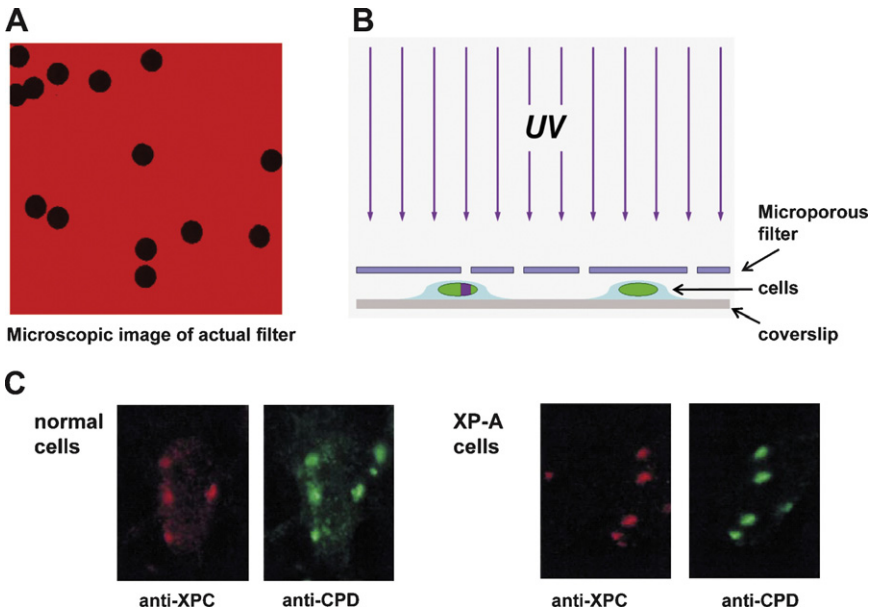

Fig. 1. Local UV-light induced DNA damage induction by irradiation through a filter. (A) Microscopic image (transmission) of a polycarbonate membrane with small (here 5 μm) pores (black filled circles). (B) Scheme of filter-assisted local UV-damage (LUD) induction in cultured cells. Cultured cells (light blue shapes, with green nuclei) adhered to a glass surface (grey), were covered with a microporous filter that shields the cells from UV-light (purple arrows), except at the position of the pores (purple bar in nucleus). C, Immunofluorescent analysis of NER proficient (left panel) and XP-A (XP25RO) (right panel) human fibroblasts irradiated with 30 J/m^2 UV-C through 3 μm filters, fixed 15 min after exposure and stained with anti-CPD antibodies (green) and anti-XPA antibodies (red).

quent immunofluorscent detection of UV-induced photo-products and NER factors provided a simple though versatile tool to visualize NER factor accumulation at these local UV-damaged (LUD) sites [22–24]. This method allowed, for the first time, the analysis of the interdependence and subsequent assembly order of NER factors into functional NER complexes within intact mammalian cell nuclei. Within a systematic analysis of NER factor binding to LUD, taking advantage of available genetically defined NER mutant cell lines, Volker et al. were able to determine the sequential steps of NER complex assembly *in situ* [24].

The impact of this seminal paper was not only restricted to the possibility of visualizing NER protein binding to damaged sites *in situ*, but also greatly contributed to finally resolve a dispute within the NER field, i.e. determination of the damage recognition activity within NER. For sometime two opposing models for damage recognition and thus initiation of the NER process were postulated based on *in vitro* experiments: in one model it was proposed that XPA with RPA are the first damage recognizing factors [25], whereas Sugasawa et al. had already provided evidence that DNA binding by XPC-HR23B initiates NER prior to XPA action [26]. Volker et al. showed that the *in situ* assembly of NER factors, including XPA, appeared to be strictly dependent on functional XPC, whereas in a reverse experiment XPC was perfectly capable of loading to LUD in the absence of XPA (Fig. 1C). I specifically emphasize this example, as it is a clear illustration of the power of this cell biological approach to unambiguously determine the sequential steps in NER complex assembly, using the mammalian cell nucleus as the best imaginable test-tube.

4. Laser-assisted Local DNA damage induction

In parallel more advanced procedures were developed to locally inflict DNA damage in living cultured cells, using focused laser-beam irradiation (summarized in [27]). This approach is more flexible than a shielding-based procedure as it allows introduction of DNA damage at a user-defined sub-nuclear area. In addition, when microscopes were equipped with this set-up, it allows direct measurements of the very early DNA damage response in living cells, particularly in cells expressing auto-fluorescent marker proteins (see below). Initially, long wavelength UV (UV-A; 405 nm) laser irradiation was combined with the DNA binding agent Hoechst and the nucleotide analog 5-bromo-2′-deoxyuridine (BrdU) [28]. The Hoechst dye serves as photo-sensitizer that significantly enhances the photolytic reaction of BrdU containing DNA to induce DNA double-strand breaks (DSBs) [29]. In due time different variants were developed with either omitting Hoechst [30,31] or BrdU [32]. These methods are currently widely used in the DSB-repair field and have significantly contributed to the cell biological level of understanding DSB repair, which has culminated into a generic spatio-temporal classification of DSB-associated DDR factors [33]. The presence of photo-sensitizers however, creates a large variety of different poorly defined DNA lesions [27] and has resulted in conflicting interpretations of the localization of the telomere binding protein (TRF2) to DSBs [32,34]. Although, UV-A irradiation in combination with Hoechst induces a large variety of DNA lesions including CPDs, surprisingly no 6-4PPs were detected [27] and shows that this method is not suited to study *in situ* NER.

Alternatively, differently tuned intense laser irradiation with 365 nm without photo-sensitizers has been used to study the DDR *in situ* [35]. This method produces a wide range of different lesions, including oxidative base damage, single-, and double-strand breaks and has provided important novel insight into the spatio-temporal organization of BER, single-strand break repair and DSB repair [35–37]. More relevant for *in situ* NER research was the notion that non-linear pulsed near-infrared (800 nm) laser micro-beam irradiation also produces CPD photo-lesions [38]. This so-called multi-photon irradiation induces however a wide range of DNA lesions with relatively high local concentrations [27] and even allowed visualization of non-homologous end-joining factor recruitment in living cells [39] that were previously not found to be accumulated on laser-assisted local DNA damage [33]. Perhaps the most appropriate local DNA damaging tool for NER was the development of a microscope setting on which a UV-C (266 nm) laser was implemented with all-quartz optics [27]. This local *in situ* DNA damaging device produces ‘clean’ NER-specific lesions, under

conditions that allow easy monitoring of fluorescent marked NER factors (see below).

5. The green fluorescent protein revolution

Local UV-damage induction and subsequent time-resolved analysis of appearance and disappearance of specific NER factors on the damaged spots provides important information on the reaction kinetics of NER in intact cells. This method does not however allow the analysis of the dynamic properties of the individual participating proteins. Further exploitation of the cell as test tube to analyze biological processes thus requires additional tools. The still increasing sophistication of live cell microscopy, particularly technological advances of confocal microscopes in terms of resolution, image acquisition speed, sensitivity and spectral opportunities provided by the more available (or even tuneable) lasers and fluorescent bioprobes and markers has tremendously boosted cell biological applications. The most important technological innovation however is the availability of genes encoding for autofluorescent marker proteins such as the green-fluorescent-protein (GFP) that has really revolutionized cell biology. Cloning of GFP in frame with genes of interest and subsequent expression of the encoded fusion protein in different cell types has opened previously unexpected research avenues. Using this procedure, location and locomotion of fluorescently-tagged proteins can now simply be monitored in their native environment by non-invasive fluorescence microscopy. Appreciation of the broad application possibilities and acknowledgement of the impact that this invaluable tool had on life sciences is illustrated by awarding the Nobel prize for Chemistry in 2008 to Drs. Shimomura, Chalfie and Tsien for their contribution to the discovery, cloning and subsequent development of this highly versatile live cell protein marker [40–43].

Although GFP-tagging and high resolution confocal imaging appeared as promising tools to study NER in living cells, initial live cell imaging of the first GFP-tagged NER factor ERCC1 did not create much excitement as no specific sub-cellular structures nor dynamic relocalization upon DNA damage induction (UV-irradiation) was observed [10]. A similar homogeneous nuclear distribution and lack of UV-induced relocalization was observed for the GFP-tagged XPA protein (Fig. 2A) [10,44]. Absence of any visible translocation of ERCC1-GFP was not due to a functional interference by the added tag, as the tagged protein appeared fully functional in NER. ERCC1-GFP was expressed in CHO that carry an inactivating *Ercc1* mutation rendering them hypersensitive to UV-light. Expression of the fusion protein fully corrected this UV-sensitivity, showing its biological activity.

6. Photobleaching (FRAP): a handy tool to study NER dynamics

To further investigate the function of ERCC1-GFP in NER 'fluorescence recovery after photo-bleaching' (FRAP) [45] was applied. The FRAP procedure is based on the physical property of any fluorescent dye that upon extensive irradiation with the excitation wavelength a fluorophore enters a permanent non-excitable status, known as photo-bleaching. Usually photo-bleaching is considered as an unwanted side effect of fluorescence imaging by microscopists, however in FRAP this property is being exploited to determine protein mobility. FRAP was originally developed to measure two-dimensional lateral Brownian motion in cell membranes [45], where fluorescence recovery within a previously bleached spot was explained by the replacement of bleached molecules with fluorescent ones in that spot. The speed at which this fluorescence recovery occurs is than a measure for the diffusion rate. Particularly after the introduction of GFP in cell biological research, the FRAP application has become common practice and in due time many different variations to the common theme have emerged, reviewed in [12,46–50]. Within the 'FRAP world' the most popular variation is to simply bleach a small area in the cell (spot) and subsequently determine the recovery of fluorescence in that previously bleached spot. This procedure measures fluorescence recovery in only a small volume and thus contains limited fluorescent information (low photon counts). With low levels of GFP-tagged protein expression (generally preferred to match with normal or physiological expression levels; see below) this procedure makes it difficult to obtain statistically reliable quantification. To circumvent this, a slightly adapted procedure was developed in which a small strip spanning the nucleus was photo-bleached (Fig. 3A and B) [12], the subsequent fluorescence recovery measurement can be obtained form a larger volume and thus contains more information.

Applying FRAP to ERCC1-GFP expressing cells revealed first of all that this protein is moving very fast through the nuclear space with a speed that corresponds to free diffusion in highly dense and viscous medium [10]. This was actually the first report that a functional nuclear protein has a very high mobility in the living mammalian cell nucleus, which was quickly confirmed by other studies [51]. In both the Houtsmuller and Phair papers evidence was provided that most non-chromatin bound nuclear proteins roam the nuclear space by passive diffusion, following simply Einstein's equation that mathematically describes Brownian motion [52]. Interpretation of FRAP data is however more complex than superficially anticipated. Initially, mobility parameters were simply derived by fitting experimentally obtained FRAP curves to mathematical models that describe two-dimensional diffusion [45]. As diffusion occurs in three dimensions more complex equations were required to describe mobility in a 3D-space. In practice complete free mobility of large biomolecules is restricted within cell nuclei by 'molecular crowding' caused by dense protein concentrations, nuclear particles (such as speckles, Cajal bodies, PML bodies, etc.) [53–55], chromatin in variable densities, nucleoli [56], nuclear shape, lamins [57] and actin fibers [58]. Due to this so called 'anomalous diffusion' by molecular crowding simple mathematical diffusion-models will not accurately describe mobility in cell nuclei and thus hamper precise determination of a diffusion constant in living cells using FRAP of GFP-tagged proteins. However, operational parameters such as an 'effective diffusion rate' can be very meaningful for comparing different proteins in the same experimental system or compare the dynamic behavior of a protein under varying conditions; e.g. in the presence or absence of DNA damage, or at different times after damage induction. In the last couple of years more sophisticated mathematical models have emerged to fit experimentally-derived FRAP data with increasing accuracy [47,59,60]. An alternative method, using computer-aided fitting to a Monte-Carlo model in which diffusion rates and different amounts of transient binding (with different affinities) can be varied, turned out most practical [50,61]. In this model parameters of actual FRAP are mimicked, these include: the size and shape of a nucleus; shape of the photo-bleaching laser cone; random Brownian motion with a step size and time frame corresponding to a confocal scan; and binding and releasing to an immobile structure (DNA). With this procedure reproducible comparative operational mobility parameters such as effective diffusion constant (D_{Eff}) and the duration (in seconds) and amount (expressed in percentage of the total amount of protein under investigation) of a (transient) immobile fraction can be determined.

7. FRAP analysis of GFP-tagged NER factors

Within a few years following the first report a number of studies appeared that describe the dynamic properties of GFP-

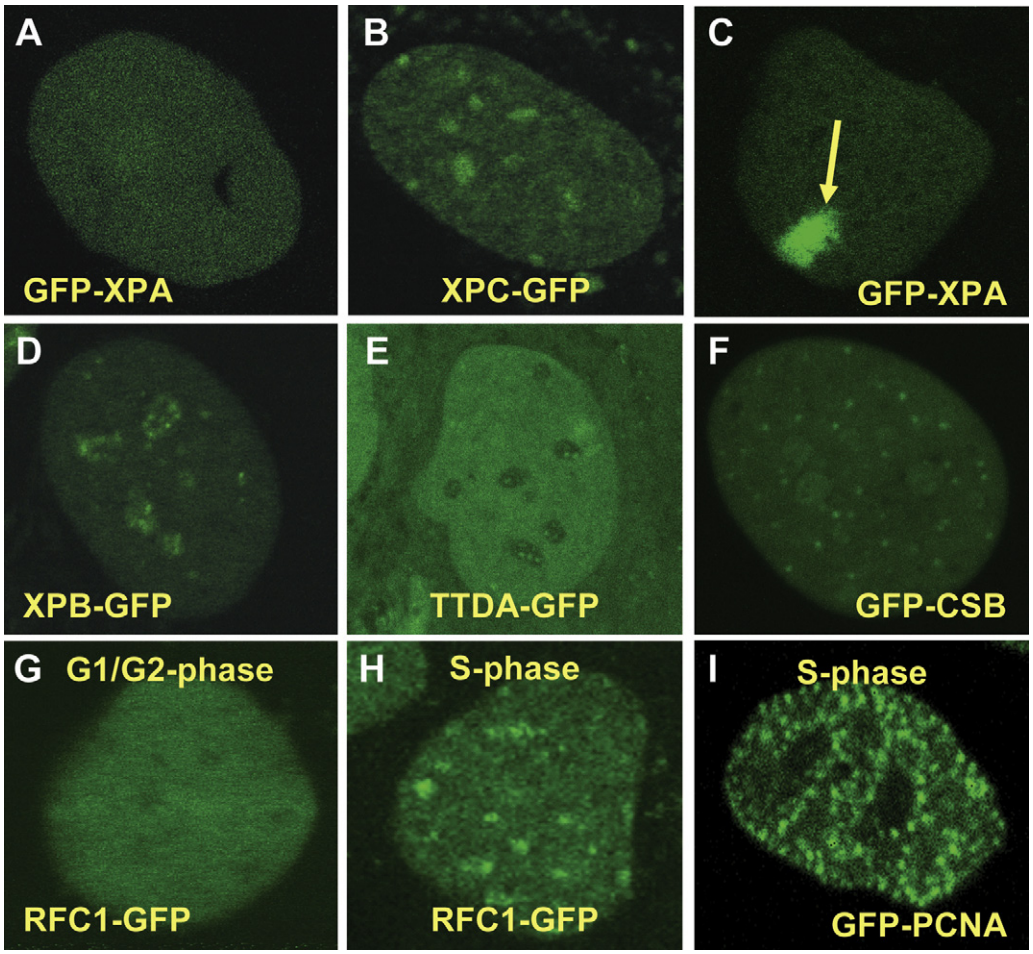

Fig. 2. Confocal fluorescent images of living mammalian fibroblasts expressing different NER factors. (A) Homogenously distributed GFP-XPA in human XP-A patient cells (XP2OS) [44]. (B) XPC-GFP is enriched in heterochromatic areas, in mouse embryonal fibroblasts ($Xpc^{-/-}$) [73]. (C) GFP-XPA accumulated at local UV-damage (arrow) generated by irradiation through a filter [44]. (D) Nuclear speckles of XPB-GFP, expressed in XP-B patient (XPCS2BA) cells [74]. (E) TTDA-GFP is localized in the nucleus (including nucleolar speckles) as well as in the cytosol when expressed in human TTD1BR (TTD-A) cells [67]. (F) Expression of GFP-CSB in CS-B deficient (CS1AN) human fibroblasts resulted in nucleolar accumulation and speckles in throughout the nucleoplasm [77]. (G and H) Homogenous distribution of RFC1-GFP in G1/G2-phase (G) and enriched in replication foci in S-phase (H) when expressed in wt human fibroblasts (MRC5) [95]. (I) Typical replication foci localization of GFP-PCNA in S-phase CHO cells [98].

tagged NER factors in mammalian cell nuclei. Several GFP-tagged NER factors, including DDB1, DDB2, XPA (Fig. 2A), ERCC1, XPF, and XPG exhibited a homogeneous distribution throughout the nucleus [44,62–65] both in the presence and absence of DNA damage (exceptions to this distribution will be discussed below). This pattern shows that the distribution of these NER factors is not restricted to certain subnuclear regions and suggests free access to even densely packed heterochromatic areas. Moreover, it also suggest that active NER is not confined to (or does not require) specific structures such as repair by homologous recombination that occurs in IRIF.

Fitting the data from the experimental FRAP curves (Fig. 3D) of the above mentioned proteins to Monte-Carlo simulated diffusion profiles [61] showed a good fit when only differential diffusion rates were simulated as variable parameter. These data suggest that in the absence of DNA damage the majority of NER factors move freely through the nucleus. Analysis of inert molecules with increasing molecular weight (i.e. fusions between GFP) showed a clear relationship between molecular size (radius of gyration) and diffusion speed [61]. The obtained FRAP-curves of the different GFP-tagged NER factors showed that each factor moved with its own specific rate (Fig. 3C and D) and thus provides a rough estimate of the molecular size with which these factors move through the nucleus. Free diffusion fits well with the observed homogeneous distribution. However, some NER factors do not exhibit complete free diffusion, these will be discussed later. FRAP studies also provide information on the composition on protein complexes within intact cells. For example ERCC1-GFP and XPF-GFP present a virtual similar mobility in mammalian cell nuclei [61] in line with the known complex formation between ERCC1 and XPF [66]. In addition, the different TFIIH sub-units appeared to move through the nucleus with a similar mobility [67] and the diffusion rate is significantly different than that of XPG [65], although it was suggested that TFIIH and XPG reside in the same complex [68]. The notion that TFIIH and XPG exhibit different mobilities does not exclude that a small fraction of these proteins are part of the same complex, but only shows that the bulk of the proteins move through the nucleus as separate molecules. The concept of individual moving NER factors contrasted however to an earlier very appealing model, based on isolation of NER factors from yeast cell nuclei, in which it was postulated that most NER-factors reside in a holo-complex, dubbed "the nucleotide excision repairosome" [69]. It was proposed that this holo-complex forms the functional unit within NER. The repairosome was an attractive model as it suggested that a functional repair unit is prepared for action to attack DNA lesions, like a fully armed and manned fire-engine ready to swiftly react to accidents. However, based on the FRAP studies in living cells and the observed interdependence of certain NER factors to bind to

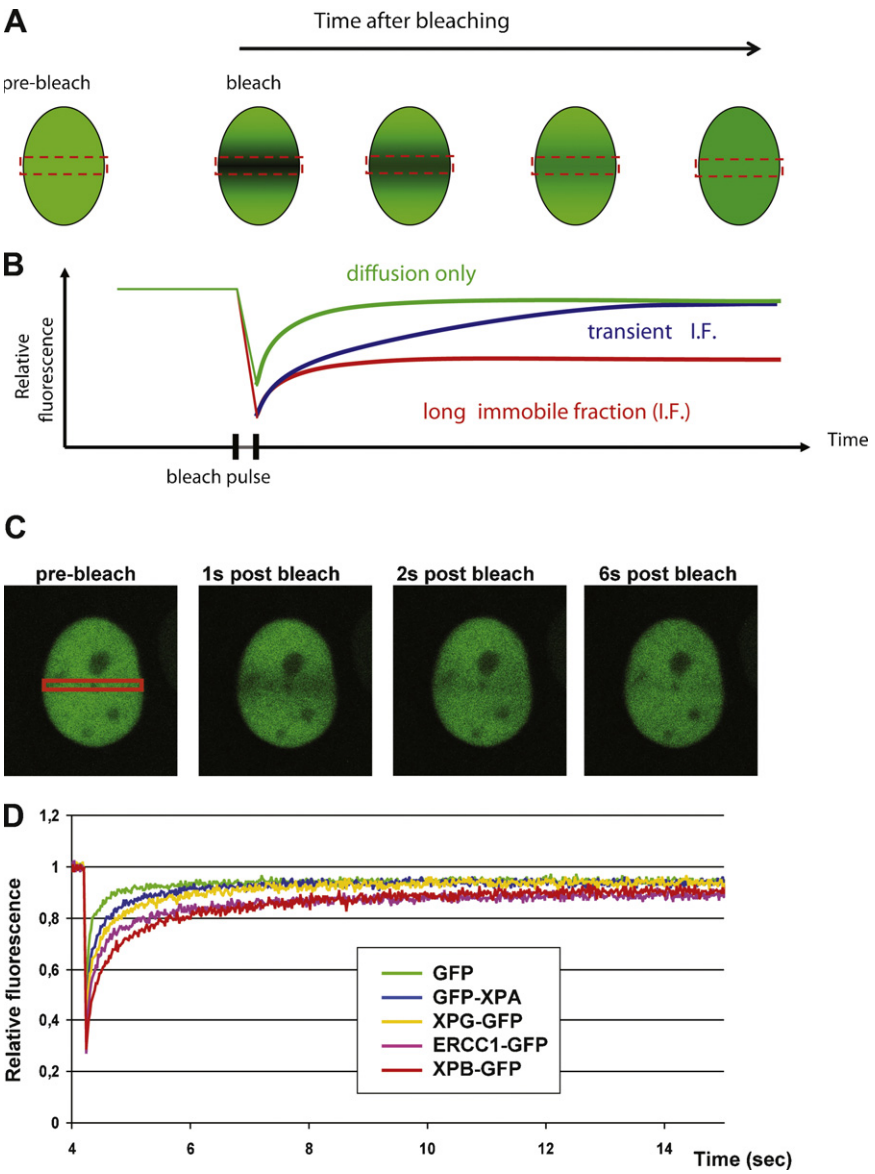

Fig. 3. Principle of fluorescence recovery after photobleaching (FRAP). (A) Model of Strip-FRAP procedure. The left green sphere represents a GFP expressing mammalian cell nucleus prior to photobleaching, the dotted red box indicates the location of the bleach area. The sphere to the right shows the bleached strip (black area), and the further spheres to the right showed the recovery of fluorescence (gradual regain of the green fluorescence) in time. (B) Fluorescence recovery plot in which the relative fluorescence (fluorescence after bleaching, divided by the fluorescence before bleaching) set at "1" is plotted against time. The green line represents quick recovery of free diffusing proteins; the red line shows an incomplete recovery caused by a long lasting immobilization of (a part of) GFP-tagged molecules; the blue line represents an intermediate situation in which part of the molecules are shortly immobilized causing in initial incomplete recovery with a secondary full recovery. (C) Images of XPG-GFP expressed in human XP-G patient (XPCS1RO) cells [65], showing typical examples of different stages during Strip-FRAP. The red box shows the bleached strip. (D) Experimental derived FRAP curves of different GFP-tagged NER factors.

LUD [24] another model was favored in which each of the individual NER factors (not pre-assembled) bind separately to the growing NER complex that resulted eventually in a functional NER machine [64,70]. This mode of lesion-bound complex formation was further corroborated by the unique assembly rates of each of the factors [71].

Although the above described method of GFP-tagging and subsequent FRAP analysis provides novel insight in the dynamic properties of individual NER components, it should be noted that the presence of the rather bulky tag (GFP is a globular protein of around 27.5 kDa) influences the mobility of the protein to which it is fused. Thus this tagging-derived intrinsic inaccuracy will (slightly) deviate from the actual speed of molecular movement of the non-tagged proteins. Obviously, the effect that a GFP-tag has on the hydrodynamic radius will be bigger for the smaller proteins not residing in a complex (e.g. XPA) and will only marginally affect the diffusional properties of larger complexes (e.g. TFIIH).

8. NER caught in action

GFP-tagged NER factors in combination with sub-nuclear UV-damage inducing procedures provided a direct visualization tool of NER complex formation. In addition, this procedure allowed the direct quantification of NER factor assembly kinetics and further confirmed that each of the NER factors are independently recruited to LUD [64,71,72]. As expected factors that act early in the process reach steady-state significantly faster than late acting factors (Fig. 4A).

FRAP analysis of different GFP-tagged NER factors after UV-irradiation revealed a significant incomplete fluorescence recovery

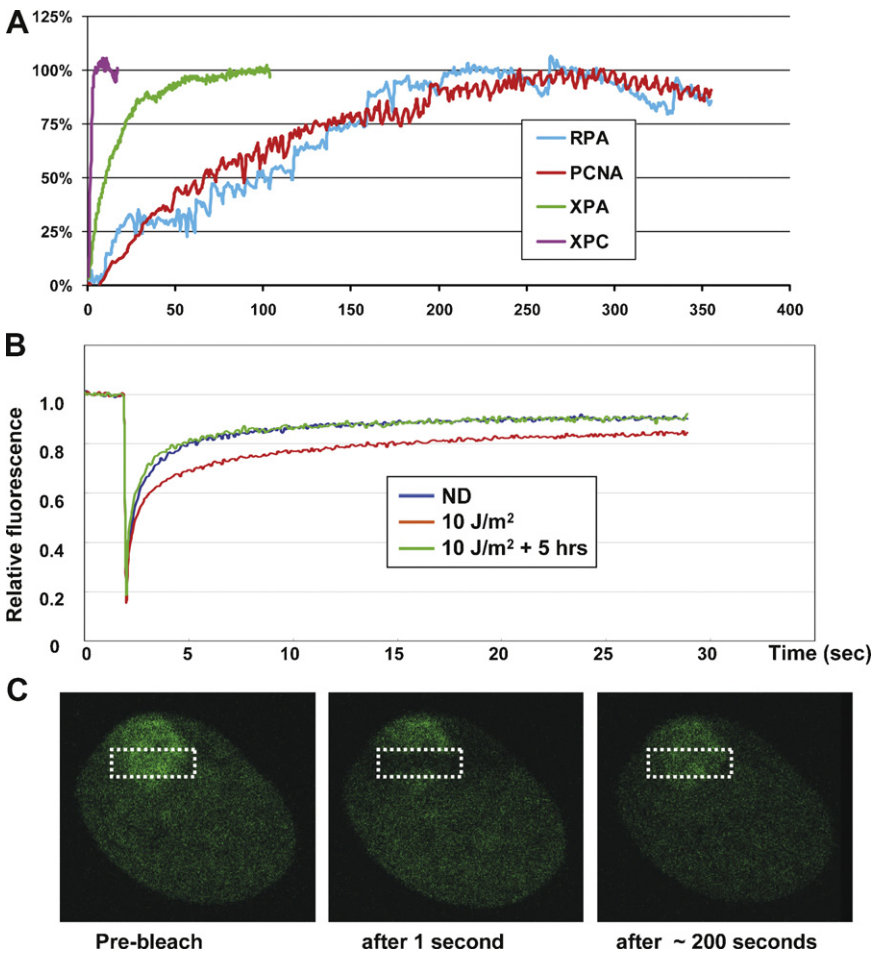

Fig. 4. Binding kinetics of NER factors to local UV-damage. (A) Assembly kinetics of different NER factors, as indicated in the inset, shows a clearly distinct binding rate of factors implicated in different stages of the NER reaction. (B) Strip-FRAP experiment to determine the amount of proteins being engaged in NER. Incomplete recovery of XPB-GFP (red line) directly after a moderate high dose of UV as compared to the non challenged cells (blue curve; ND for not-damaged). Five hours after irradiation the FRAP curve (green line) has returned to the initial undamaged situation. (C) FRAP on local UV damage (LUD) to determine binding time in chromatin-bound NER complexes. Part of the XPB-GFP accumulated at LUD is bleached and the subsequent time required for full recovery provides an estimate of the average binding time of a factor at damaged DNA.

[10,44,62,63,65,72–74] (Fig. 4B). This absence of full recovery is explained by the transient binding (Fig. 3B) of the protein to the chromatin-bound (active) NER complexes, assuming that chromatin represents a virtually immobile structure in the nucleus. Obviously, chromatin is not static but with respect to the high mobility of the GFP-tagged proteins and the time-frame in which the measurements were performed the only restricted chromatin-fiber motion is considered as immobile. The amount of immobilization is, in part, linear with respect to the applied UV-dose, i.e. more immobilized protein with increasing UV-dose. Moreover, this transiently bound pool of GFP-tagged NER factors gradually disappeared within a few hours after damage induction (Fig. 4B), approximately in the same time-frame as the 6-4PP clearance by NER. Together these data strongly argue that the observed transient immobilization reflects actual participation of these factors in NER. For this quantitative analysis of NER it is extremely important to know and control the expression level of the GFP-tagged NER protein. It was noted in the previous study [10] that different expression levels strongly affect the quantification of the active pool of ERCC1-GFP (i.e. the fraction of ERCC1 that is actively engaged in NER and thus temporarily immobilized). Careful selection of clones that express physiological amounts of the tagged protein is thus required. Single cell analysis of expression levels to avoid quantification on individual over-expressing cells further fine tunes quantification [44,72].

The amount of immobilization and the UV dose-dependency is strikingly different between the different tested NER factors. Whereas the GG-NER initiating factors DDB2 and XPC can almost completely be immobilized with increasing UV-doses [63,72,73], the later factors (XPA, ERCC1 and XPG) exhibit a relatively early saturation of UV-induced immobilization [10,44,65]. The damage-verifying factor TFIIH followed a similar pattern of UV-dose dependent binding to chromatin as XPC [72]. Although DDB2 and DDB1 show a very quick and simultaneous binding in response to UV-damage induction, only DDB2 can be completely immobilized with a high UV-dose [75]. A significant fraction of DDB1 remained mobile under these circumstances, in line with the multiple engagements of this protein with other WD40-repeat containing proteins [76]. NER-dependent DDB1 immobilization is highly dynamic and appeared to be controlled by DDB2 levels. Surprisingly, XPC immobilization does not follow a linear relationship with the applied dose as DDB2 does and exhibits a biphasic character [63,72]. UV-induced XPC immobilization saturated relatively soon at around 5 J/m^2 and only above ~10 J/m^2 a further dose-dependent increase of XPC binding was observed with a subsequent second saturation around 20 J/m^2. After passing this second threshold (between 20 and 35 J/m^2) the amount of XPC immobilization further increased in a dose-dependent manner. The amount of immobilized XPC is dependent on the DDB2 levels but its peculiar biphasic saturation is

not directly explained by DDB2 and remains for the time being enigmatic.

When at steady state the locally damaged area is bleached and subsequently the fluorescence recovery in the bleached region is measured, an estimate of the mean binding time of individual NER factors to damaged DNA can be made (Fig. 4C). Alternatively, the inverse of FRAP, called FLIP (for: fluorescence loss in photobleaching), can be applied to determine the off rate of NER factors from damaged DNA [12]. In this procedure the fluorescence outside the region of interest (here LUD) is photobleached. The rate at which the fluorescence at the LUD diminishes (fluorescent molecules exchange for bleached ones) is than a measure for the speed of repair factors that move from the damaged site. With both procedures (FRAP on LUD and FLIP outside LUD) the average dwell times of most NER factors on damaged DNA was approximately 4 min, with the exception of XPC that exhibits a shorter binding time [73]. Modeling of the assembly kinetics as well as the average binding times revealed a highly dynamic and continuous exchange of NER factors until the reaction is completed [71].

9. Chromatin painting in living cells by XPC-GFP

Above, some common dynamic features of NER factors in living mammalian cells are summarized. However, as often observed in nature, once general concepts are set, more and more 'exceptions-to-the-rule' appear, such exceptions seem to hold for the distribution and mobility of NER factors as well. Simple free diffusion models did not match the experimental FRAP curves of some other NER factors, such as XPC (damage recognition in GG-NER), XPB (part of TFIIH) and CSB (TC-NER specific) [73,74,77]. Live cell analysis of XPC-GFP revealed that in contrast to the other NER factors XPC is non-homogeneously distributed in mammalian cell nuclei [73]. XPC-GFP exhibited the characteristic heterogeneous pattern of chromatin in interphase nuclei, i.e. enriched in highly condensed chromatin, similar to the GFP-tagged core Histones (though not as immobile as these) [49]. This specific localization is even clearer when the fusion protein was expressed in mouse fibroblasts that contain the characteristic microscopically discernable heterochromatic spots (Fig. 2B). In addition, XPC-GFP was even specifically located in highly condensed mitotic chromatin [73] and is strikingly different than any of the other NER factors, which were excluded from mitotic chromosomes. The colocalization with condensed mitotic chromatin further showed that this GG-NER initiating factor has access to highly condensed chromatin. Furthermore, it also suggests that it is able to associate with chromatin even in the absence of DNA damage. Subsequent FRAP analysis revealed a surprisingly slow mobility that did not match with free diffusion. Mutational analysis and modeling of FRAP data showed that the slow mobility was derived from continuous binding to (non-damaged) DNA [73,78,79], which is line with the high *in vitro* affinity of XPC for DNA. Live cell studies on XPC mutants further provided important information on how XPC interacts with damaged DNA [78,79].

10. Dynamics of TFIIH

For the XPB protein, which is part of the transcription initiation complex TFIIH [80], anomalous diffusion was easily explained by its other main cellular function, i.e. transcription initiation [74] that includes binding to gene promoters. Although FRAP curves of XPB-GFP do not correlate with free diffusion, it was not easy to deduce from these data the fraction and binding time of TFIIH molecules being bound to promoters. Using transcription inhibitors, that block initiation-dependent TFIIH promoter binding and Monte-Carlo modeling of a variant of FRAP, these parameters could be estimated [74]. This procedures combines FRAP with FLIP and allows a more accurate measurement of short binding molecules [12,50]. These short binding times were not visualized by the distribution pattern, as besides nucleolar enrichment, XPB was otherwise homogeneously dispersed in the nucleoplasm (Fig. 2D). Accumulation of TFIIH in the active centers of nucleoli was not anticipated for an RNA-polymerase II (RNAP2) transcription factor and not observed by classical immuno-fluorescence (IF) studies. However, coincidently *in vitro* experiments revealed a novel role for TFIIH in RNA-polymerase I (RNAP1) transcription [81]. The surprising notion that the enrichment of TFIIH in the active centers of nucleoli was only observed in living cells and not in IF studies was explained by the fact the TFIIH-epitopes were masked within the dense nucleolar structures upon fixation. This observation further underscores the power of live cell imaging with autofluorescent fusion proteins as it avoids potential fixation problems.

Modeling of XPB-GFP FRAP data in nucleoli and nucleoplasm suggested very short RNAP1-, and RNAP2-dependent transcription binding of ~20 and ~6 s respectively. This modeling also indicated that a relatively large number of TFIIH molecules ($\sim 3 \times 10^4$, 30% of the total amount of TFIIH) at any given time are transiently bound. Since the calculated amount of interactions even outnumbered the sum of active protein-coding genes, of which only a fraction is expected to be active at any given moment in time, a model is proposed in which only a fraction of the molecular interactions lead to a functional transcription initiation event. The above outlined kinetic modeling disclosed a highly dynamic and probabilistic nature of molecular interactions within transcription initiation in living cells. These observations, together with similar studies on other complex processes (e.g. RNAP1 [82]) has changed our static-oriented view on molecular processes. Similar highly dynamic models for NER will be discussed below.

The mobility of TFIIH mentioned here was determined by measuring the core XPB subunit. However when the more loosely associated TFIIH component XPD [83] was measured, a more complicated mobility profile was revealed [67]. The overall mobility of XPD-GFP was significantly different than that of XPB-GFP and appeared to be mainly due to a non-TFIIH associated fraction that is present both in the nucleus and cytoplasm. At least two different XPD-containing sub-complexes exist; one associated with CAK (hetero-trimer of CDK7, CCNH and MAT1; implicated in cell cycle regulation [83]) and a cytosolic complex as part of the so-called MMXD complex (MMS19 and XPD, implicated in chromosome segregation [84]). When the cytosolic and more mobile nuclear fraction of XPD was bleached (using another adapted FRAP procedure, referred to as 'FRAP-abc') the remaining nuclear fraction exhibited a similar mobility as XPB [67]. The smallest TFIIH subunit, TTDA, also showed a different mobility and distribution (Fig. 2E) than the core TFIIH subunit XPB. Although identified and isolated as a TFIIH subunit, the majority of TTDA only dynamically interacts with TFIIH and this interaction is stabilized by UV-irradiation [67]. This observation was very surprising as TTDA appeared to be crucial for TFIIH stability [85,86] and showed that even transient interactions can be important for complex stability.

11. Transcription-coupled repair

Perhaps the most striking deviating sub-nuclear distribution of the NER factors tested was observed with GFP-tagged CSB (Fig. 2F). Next to a nucleolar accumulation also foci or speckle-like structures were present in non-damaged cells [77]. Both these high local concentrations seem to be very sensitive to DNA damage induced stress as they quickly fade upon DNA damage. The foci appear to partly co-localize with splicing factors (van den Boom, unpublished observations), similar to the so-called "speckles" in which tran-

script maturation occurs [53]. However, the exact nature of these foci remains enigmatic as co-localization studies are hampered by the fact that these structures are very sensitive to changes in the cellular environment such as different fixation procedures. As for TFIIH, also a possible function for CSB in ribosomal gene expression was suggested [87], although the sub-nucleolar distribution of CSB is different than for TFIIH. TFIIH localization seems to be restricted to the so-called '*pars fibrosa*', i.e. the active RNAP1 transcription sites, whereas CSB is homogeneously distributed in the nucleolus. Mobility studies suggested that part of the relative slow mobility of CSB is derived from a continuous very short binding of CSB to elongating RNAP2 complexes [77]. This observation confirmed earlier biochemical studies in which a direct association between CSB and RNAP2 was found [88–90]. Surprisingly, only a small amount of further immobilization upon DNA damage induction could be observed at a relative long time after UV-exposure for this crucial TC-NER factor. Based on these studies a model was proposed in which CSB monitors progression of transcription by regularly probing elongation complexes and that it becomes more tightly associated to these complexes when RNAP2 is stalled at lesions and TC-NER needs to be activated. A more stable association of CSB to lesion-stalled complexes than further favors the subsequent recruitment of NER factors to these sites, as it was shown that CSB is crucial for this recruitment [91]. Localizing GFP-tagged CSB to DNA damage appeared rather difficult as only faint accumulation could be observed. Together with the small amount of UV-dependent immobilization it further argues that either only a small fraction of this protein binds to lesion-stalled RNAP2 complexes or that the interaction is so short that specific binding is not easily revealed.

A recent functional study on a newly discovered domain in CSB, i.e. a conserved ubiquitin-binding domain (UBD) [92], further provided evidence that it is likely the transient nature of the interactions that makes it difficult to visualize CSB binding in TCR complexes. GFP-CSB proteins harboring UBD deletions or point mutations, were still able to bind to TC-NER complexes (and to facilitate NER factor assembly), but showed a strongly reduced dissociation, visualized as a large immobile fraction upon UV-irradiation [92]. Next to providing important novel information on regulatory role of an ubiquitinated target in TC-NER in a dynamic molecular hand-over of TC-NER proteins, it further exemplifies the power of live cell imaging to reveal dynamic molecular processes.

12. NER associated DNA replication

Yet another step in the complete NER process is the gap-filling DNA synthesis. Although, this step is crucial to complete the repair reaction, it is considered less specific for NER, as it involves genuine DNA synthesis factors as PCNA, RPA, RFC and different DNA polymerases (δ, ε and κ) [93–96] that are also pivotal for replicative DNA synthesis. For this reason, and because repair replication likely involves specific structural requirements (Gourdin, unpublished observations) as in normal replication (that proceeds in replication foci) a full summary and discussion will be too large to fit here and is beyond the scope of this review, so here only the dynamics of these proteins in NER will be shortly summarized.

The homo-trimeric sliding clamp PCNA is a crucial DNA synthesis initiation factor and was found accumulated in nuclear foci in S-phase cells (Fig. 2I). Two main studies describe the behavior of GFP-tagged PCNA in living mammalian cells. First, Spobert et al. [97] provide a detailed time-lapse imaging of GFP-PCNA on replication foci. In their study they provide evidence for a very slow turnover of PCNA molecules form replication foci and only disassemble when replication foci stop firing and novel, adjacent, replication sites were *de novo* assembled with PCNA. However, combined FLIP and FRAP studies together with single particle tracking showed that PCNA was able to exchange from individual replication foci [98]. Within NER the turnover of PCNA was even slower and remained for a very long time after UV-exposure [98]. In an attempt to dissect the PCNA function in NER from its other UV-induced DDR role in TLS (that requires PCNA ubiquitination [99]), an ubiquitination-deficient mutant (K164) of PCNA fused to GFP was used. Surprisingly, a shorter binding time in NER was revealed with this mutant, suggesting a role of ubiquitinated PCNA in NER in dissociating PCNA [98]. Only very recently a functional significance of this observation was provided by the notion the TLS polymerase; DNA polymerase κ (DNAPolκ) functions in NER and requires ubiquitinated PCNA [94].

In comparison with PCNA an unexpectedly different kinetics in replication and in NER-dependent DNA synthesis was observed for the clamp-loader RFC [95]. The largest subunit (RFC1 or RFCp140) of this hetero-pentameric complex was fused to GFP and expressed in human fibroblasts. RFC1-GFP is homogenously distributed in cell nuclei in the G1 and G2 phase of the cell cycle (Fig. 2G) and exhibits the typical focal pattern in S-phase (Fig. 2H) as many other replication factors. Next to the notion that this study provided the first direct evidence that RFC is implicated in NER *in vivo* an unexpected highly dynamic interaction with both replication foci and NER sites was observed and sharply contrasts with the relatively long interaction of PCNA with replication and repair sites [95,98]. Remarkably when repair DNA synthesis was inhibited by HU and AraC, RFC remains targeted to repair sites. Since RFC will not be used here to load PCNA under these conditions, as the clamp is already loaded and remains bound when synthesis is inhibited, these data suggest an additional role for RFC beyond PCNA loading [95].

13. Conclusion

Essentially all knowledge on the functioning of basic cell biological processes, such as replication, transcription and DDR, has been gathered from *in vitro* cell-free systems. Only recently, with the aid of GFP-tagging and advanced confocal microscopes novel insight has been gained on the dynamic interplay (e.g. mobility and binding-dissociation equilibria) of a number of factors implicated in chromatin-associated processes. These studies have culminated into a general model in which the majority of DNA-transacting proteins freely diffuse through the nucleus and stochastically bind to active sites such as DNA lesions, promoters, or replication foci to finally create a functional complex. The dynamic assembly of different factors will eventually occur with only low probability, but allows flexible sharing of components, regulation at multiple levels and several quality control checks [82,100,101]. Further mathematical modeling of NER factor dynamics and NER-complex formation based on experimentally derived kinetic parameters revealed a highly dynamic and continuous exchange of repair factors even during a single repair reaction [71]. Within this model even a random and reversible order of assembly was suggested, beyond the initial damage recognition step, to allow efficient repair. Genetic data however, suggest a certain sequential order of NER factor assembly, e.g. XPG only becomes stably integrated with a functional TFIIH [65] and argue that non-reversible energy requiring steps are implicated in NER complex maturation and damage verification. This concept of kinetic proofreading is taken into account in the model and was suggested as a driving force in NER progression [71]. Although, this model is not complete yet, as several NER regulating factors are not included it turned out to be a valid predictive and testable model, waiting to be falsified or refined by more accurate or more complete models.

14. Future perspectives

Obviously, care has to be taken with generalizing this highly dynamic NER model as the kinetic data that formed the experimental input of this modeling were all derived from measurements in cultured cells. Cultured cells are under constant stress (e.g. atmospheric oxygen) and usually in a highly replicative status. Moreover, physiological processes critically depend on the cellular context or micro-environment (cell-cell contacts with neighbouring cells, extracellular matrix, etc.). In addition, within larger animals, more than 90% of the somatic cells are in a non-proliferative status, thus making extrapolations to the actual *in vivo* situation even more delicate. These notions argue for the need to investigate NER in the whole organism. To acquire an integral view on the dynamic behavior of NER in diverse differentiated cells, a knock-in mouse model that expresses from its endogenous gene locus a fluorescently tagged protein was generated [102]. One of the subunits of the repair/transcription factor TFIIH, XPB, was tagged with the yellow variant of GFP (YFP), by targeted gene integration in ES cells. From these pluripotent ES cells mice were generated that express YFP-fused to XPB (XPB-YFP) under control of the normal transcription regulatory elements, providing physiological expression in all cell types. Within cultured cells it was shown that TFIIH is highly dynamic and only transiently interacts with promoters in the order of a few seconds (i.e. a "hit-and-run" dynamic model) [74]. A similar dynamic behavior was observed in highly proliferative cells in mouse tissue, such as keratinocytes in skin [102]. Surprisingly, in some highly differentiated post-mitotic tissues (e.g. neurons) TFIIH appeared almost permanently immobilized to chromatin in a transcription-dependent fashion. This suggests that a well-known and extensively studied cellular pathway, such as transcription, can have a completely different dynamic organization in different cells. The mechanistic reason for these kinetic differences of transcription regulation between different cell types remains enigmatic and will be the next challenge to reveal.

In view of these observations it remains questionable whether current concepts of NER functioning are applicable to all cell types or tissues. It is known for example that GG-NER and TC-NER play different roles in UV-defense in different cell types and developmental stages [103,104]. Moreover, a neuron-specific specialized NER sub-pathway appeared to be present, dubbed DAR (for domain-associated repair) [105]. This mouse-model will be further exploited to study NER in different cell types and during development.

Dynamic studies in living cells and, recently, in living animals allow us to study repair mechanisms in action. Together with emerging systems biological approaches, these new tools and techniques provide tremendous opportunities to reach our final goal of a full comprehension of NER in its cancer protection and anti-aging role and to understand the biological consequences of deficient NER in patients. I would like to close with the notion that a meaningful interpretation of the live cell repair dynamics and kinetics was only possible (and perhaps only makes sense) because of the tremendous amount of available genetic and biochemical information on most of the implicated factors. In this way the obtained kinetic parameters can be linked to known functions and can be incorporated into the global and still growing picture of how NER functions.

Conflict of interest

There is no conflict of interest.

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