Biochemical and Biophysical Research Communications 433 (2013) 58–65

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

# Diffusion dynamics of the Keap1–Cullin3 interaction in single live cells

Liam Baird [a], Albena T. Dinkova-Kostova [a,b,*]

[a] Jacqui Wood Cancer Centre, Division of Cancer Research, Medical Research Institute, University of Dundee, Dundee, Scotland, UK
[b] Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine, Baltimore, MD, USA

ARTICLE INFO

*Article history:*
Received 13 February 2013
Available online 26 February 2013

*Keywords:*
Chemoprotection
Cullin3
FRAP
Inducer
Keap1
Nrf2

ABSTRACT

Transcription factor NF-E2 p45-related factor 2 (Nrf2) regulates the expression of a network of genes encoding drug-detoxification, anti-inflammatory, and metabolic enzymes, as well as proteins involved in the regulation of cellular redox homeostasis. Under basal conditions, Kelch-like ECH associated protein 1 (Keap1) targets Nrf2 for ubiquitination and proteasomal degradation via association with Cullin3 (Cul3)-based Rbx1 E3 ubiquitin ligase. Various small molecules (inducers) activate Nrf2 leading to upregulation of cytoprotective gene expression. Inducers chemically modify specific cysteine residues of Keap1 which ultimately loses its ability to target Nrf2 for degradation. Dissociation of the Keap1–Cul3 complex by inducers is one possible mechanism, but evidence in single live cells is lacking. To investigate the diffusion dynamics of the Keap1–Cul3 interaction and the effect of inducers, we developed a quantitative fluorescence recovery after photobleaching (FRAP)-based system using Keap1–EGFP and mCherry–Cul3 fusion proteins. We show that Keap1–EGFP and mCherry–Cul3 interact in single live cells. Exposure for 1 h to small-molecule inducers of 4 different types, the oleanane triterpenoid CDDO, the isothiocyanate sulforaphane, the sulfoxythiocarbamate STCA, and the oxidant hydrogen peroxide which target distinct cysteine sensors within Keap1 with potencies which differ by nearly 4000-fold, does not dissociate the Keap1–Cul3 complex. As inducers cause conformational changes in Keap1, we conclude that changes in conformation rather than dissociation from Cul3 inactivate the repressor function of Keap1 leading to Nrf2 stabilization.



## 1. Introduction

Elaborate networks of cytoprotective proteins (e.g., NAD(P)H:quinone oxidoreductase-1, heme oxygenase-1) defend against the damaging effects of oxidants, electrophiles, and inflammatory agents, the principal contributors to the pathogenesis of all chronic diseases. Transcription of their genes, controlled by NF-E2 p45-related factor 2 (Nrf2), can be upregulated (induced) by a variety of synthetic and natural agents (inducers) [1]. At basal state, the levels of Nrf2 are maintained low largely due to binding to Kelch-like ECH associated protein 1 (Keap1), which targets Nrf2 for ubiquitination and proteasomal degradation via association with Cullin3 (Cul3)-based Rbx1 E3 ubiquitin ligase. Inducers react and chemically modify specific cysteine residues of Keap1 which consequently loses its ability to target Nrf2 for degradation [2,3]. This leads to the stabilization and nuclear translocation of Nrf2 where it binds as a heterodimer with small Maf proteins to antioxidant response elements (AREs) and activates transcription. Induction of cytoprotective proteins protects against oxidants and carcinogens [4]. However, persistent upregulation of Nrf2-dependent genes is frequently exploited by cancer cells, promoting their survival and resistance to chemotherapy [5,6]. The Cancer Genome Atlas Research Network identified the occurrence of mutations in *NRF2*, *KEAP1*, or *CUL3* in 34% of 178 lung squamous cell carcinomas [7]. Thus, detailed mechanistic understanding of the regulation of the Nrf2 activity is critical for the development of chemoprotective and chemotherapeutic strategies.

Several models have been proposed to explain the Keap1-mediated regulation of Nrf2 [4]. One of the first experiments to recognize the role of Cul3 in the Keap1-dependent repression of Nrf2 found that Keap1 and Cul3 were dissociated by the inducers *tert*-butyl hydroquinone (tBHQ) and sulforaphane (SFN) [8]. Importantly, Cys151 of Keap1 was required for this effect. Dissociation of Cul3 from Keap1 was also reported to occur upon exposure to eicosapentaenoic acid and N-iodoacetyl-N-biotinylhexylenediamine (IAB) [9–11]. Using recombinant proteins, it was shown that IAB could both prevent Keap1 from binding to Cul3 as well as dissociate

*Abbreviations:* CDDO, 2-cyano-3-,12-dioxooleana-1,9 (11)-dien-28-oic acid; Cul3, Cullin3; FRAP, fluorescence recovery after photobleaching; Keap1, Kelch-like ECH associated protein 1; NQO1, NAD(P)H:(quinone acceptor) oxidoreductase-1 (EC 1.6.99.2); Nrf2, NF-E2 p45-related factor 2; SFN, sulforaphane; STCA, sulfoxythiocarbamate alkyne.

* Corresponding author. Address: Division of Cancer Research, Medical Research Institute, James Arrott Drive, Ninewells Hospital and Medical School, Dundee DD1 9SY, UK.
*E-mail address:* a.dinkovakostova@dundee.ac.uk (A.T. Dinkova-Kostova).

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http://dx.doi.org/10.1016/j.bbrc.2013.02.065

pre-formed Keap1–Cul3 complexes [10]. Thus, dissociation of the Keap1–Cul3 complex by inducers is one possible mechanism leading to inhibition of Nrf2 ubiquitination and induction of cytoprotective gene expression. The current data to support this model have been obtained using cell populations or recombinant proteins *in vitro*, and additional approaches are needed to examine the dynamics of the interaction between Keap1 and Cul3. The use of quantitative live cell imaging is ideally suited to study protein–protein interactions in individual cells and in their correct physiological environment [12,13]. In this study, we developed a quantitative fluorescence recovery after photobleaching (FRAP)-based system which allowed us to investigate the dynamics of the Keap1–Cul3 interaction in single live cells.

## 2. Material and methods

### 2.1. Chemicals and inducers

All reagents were from Sigma–Aldrich (Poole, Dorset, UK) unless specified. Inducers were from: SFN (LKT laboratories Inc., St. Paul, MN, USA), STCA (a generous gift from Dr. Young-Hoon Ahn and Dr. Philip A. Cole, Johns Hopkins University, USA), 2-cyano-3-,12-dioxooleana-1,9 (11)-dien-28-oic acid (CDDO) (a generous gift from Dr. Michael B. Sporn and Dr. Tadashi Honda, Dartmouth College, USA), $H_2O_2$ (Sigma). In order to gain a thorough understanding of the mechanism of Nrf2 activation, we chose inducers which target different cysteine sensors of Keap1. Thus, SFN was used as an inducer specific to the Cys151 sensor of Keap1 [14–16], whereas STCA was used to specifically target the Cys273, Cys288, and Cys613 sensors [17]. In addition, because the inducer concentrations that are required to stabilize Nrf2 vary over several orders of magnitude, we used inducers with a wide range of potencies. Thus, CDDO (which may target Cys226 of Keap1 [18]) was selected as a highly potent inducer [19] and used at a concentration of 0.1 μM. In sharp contrast, the weak inducer $H_2O_2$ [20,21] was applied at a concentration of 400 μM.

### 2.2. Cloning

The pmEGFP-C1, pmCherry-N1 and pmCherry-C1 were from Clontech. The mouse Nrf2 and Cul3 cDNA vectors, and the EGFP–Keap1 vector were all kind gifts from Dr. Michael McMahon and Dr. John Hayes (University of Dundee). The general cloning strategy employed to generate the fusion proteins was the same for all constructs. The cDNA encoding the gene of interest was amplified using PCR to introduce the restriction enzyme consensus sequences required for cloning. The PCR product was purified on an agarose gel, extracted and digested, along with the destination vector, with the appropriate pair of restriction enzymes (KpnI and AgeI for Nrf2–mCherry and pmCherry-N1, or XhoI and KpnI for mCherry–Cul3 and pmCherry-C1). The digested vector and PCR product were purified again from an agarose gel, before being ligated together and transformed into competent bacteria. Individual transformed colonies were then picked and grown overnight in 5-ml mini-cultures, from which the plasmids were purified and sequenced. The following primers were used to clone: (i) Nrf2 into the pmCherry-N1 vector: FORWARD primer: CCGCCGCTACCATGATGGACTTGGAGTTGCCACCGCCAG, and REVERSE primer: ATCCTCCACCGGTTTGTTTTTCTTTGTATCTGGCTTCTTG; (ii) Nrf2 into the pEGFP-C1 vector: FORWARD primer: TCCGCTCGAGCAATGATGGACTTGGAGTTGCCACCGCCAG, and REVERSE primer: TCCTCGTCGACCTAGTTTTTCTTTGTATCTGGCTTCTTG (iii) Cul3 into the pmCherry-C1 vector: FORWARD primer, GCCGCTCGAGCTATGTCGAATCTGAGCAAAGGC, and REVERSE primer: GAAGGTACCTTATGCTCACTATGTGTATAC; and (iv) Keap1 into both the EGFP-N1 and pmCherry-N1 vectors: FORWARD primer: ATTGGTACCATGCAGCCCGAACCCAAGCTTAG, and REVERSE primer: ATGAAGACCGGTGCTTTTGCTGCTGCCTCTTTAGCGGCTGCTTCTGCGCAGGTACAGTTTTGTTGATC.

### 2.3. Cell culture

HEK293 cells were grown in α-MEM supplemented with 10% heat-inactivated fetal bovine serum (Gibco) on gelatin-coated plastic dishes in 5% $CO_2$ at 37 °C. For microscopy experiments, 200,000 cells were seeded onto Willco glass bottom dishes.

### 2.4. NQO1 assay

Cells (200,000 cells per well) were grown in 6-well plates for 24 h. The medium was removed and fresh medium containing inducers or vehicle (acetonitrile, 0.1% v/v) was added, and the cells were grown for a further 48 h. The enzyme activity of NQO1 was determined in cell lysates with menadione as a substrate [22].

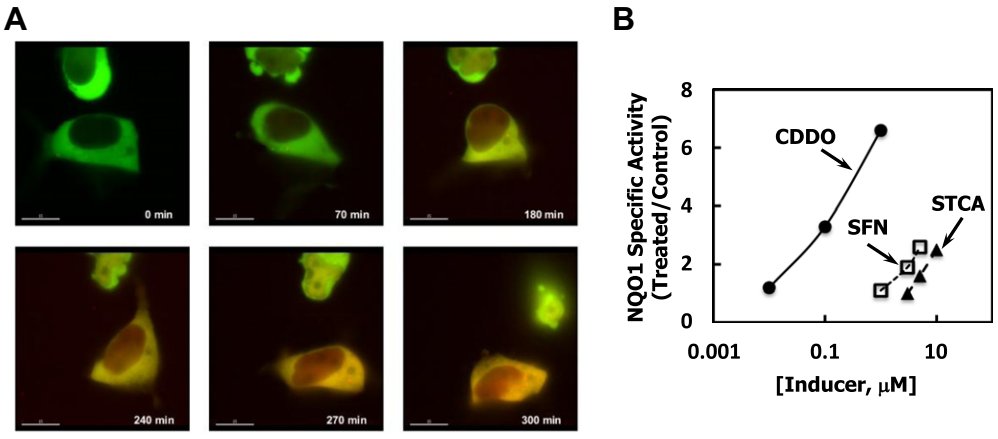
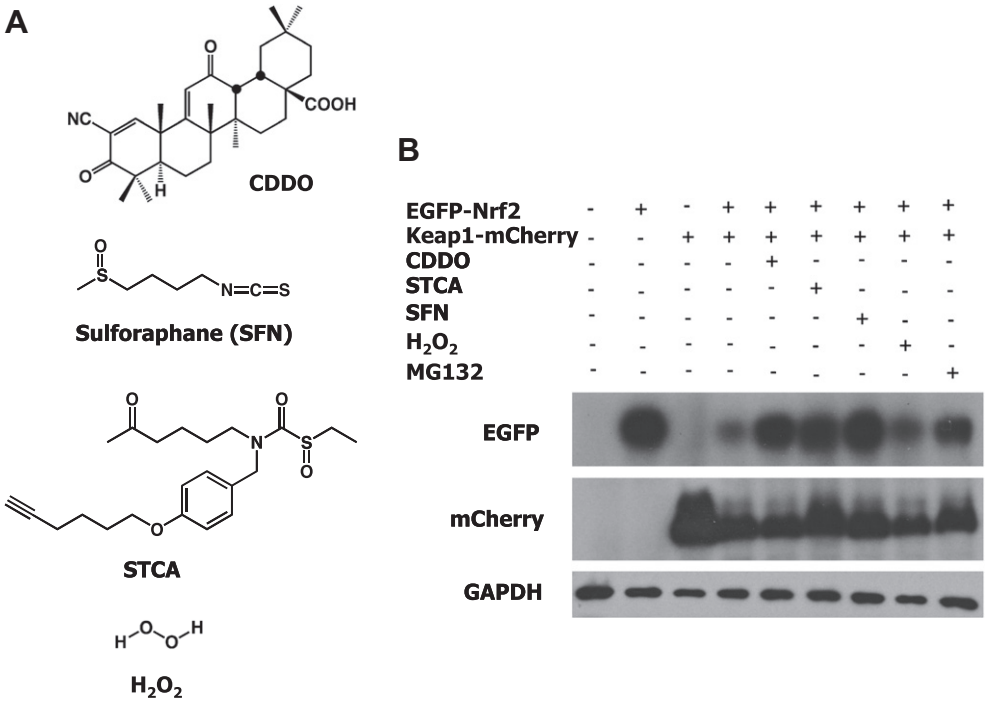



**Fig. 1.** EGFP–Keap1 is functional both as a substrate adaptor for mediating Nrf2 degradation as well as an inducer sensor. (**A**) Nrf2–mCherry and EGFP–Keap1 constructs were transfected into HEK293 cells and imaged in a time-lapse experiment. At time 0, EGFP–Keap1 is localized in the cytoplasm and Nrf2–mCherry is undetectable. After addition of CDDO (0.1 μM), Nrf2 becomes stabilized as evident by the increase in red fluorescence. Scale bar = 15 μm. (**B**) HEK293 cells ($2 \times 10^5$ per well) were grown in 6-well plates for 24 h. The medium was removed and replaced with fresh medium containing inducers, the cells were grown for a further 48 h, and the NQO1 activity was determined (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).




C

| | Inducer | T½ (s) | N | SD | T-test |
|---|---|---|---|---|---|
| Keap1-EGFP + mCherry | None | 0.290 | 41 | 0.073 | |
| Keap1-EGFP + mCherry-Cul3 | None | 0.347 | 15 | 0.098 | p = 0.022 |
| | | | | | |
| Keap1-EGFP + mCherry-Cul3 | None | 0.374 | 18 | 0.085 | |
| Keap1-EGFP + mCherry-Cul3 | 0.1 μM CDDO | 0.372 | 30 | 0.096 | p > 0.05 |
| | | | | | |
| Keap1-EGFP + mCherry-Cul3 | None | 0.320 | 20 | 0.075 | |
| Keap1-EGFP + mCherry-Cul3 | 5 μM SFN | 0.334 | 22 | 0.053 | p > 0.05 |
| | | | | | |
| Keap1-EGFP + mCherry-Cul3 | None | 0.347 | 15 | 0.098 | |
| Keap1-EGFP + mCherry-Cul3 | 10 μM STCA | 0.367 | 27 | 0.090 | p > 0.05 |
| | | | | | |
| Keap1-EGFP + mCherry-Cul3 | None | 0.416 | 29 | 0.087 | |
| Keap1-EGFP + mCherry-Cul3 | 400 μM $H_2O_2$ | 0.385 | 20 | 0.061 | p > 0.05 |

**Fig. 2.** Keap1–mCherry efficiently targets EGFP–Nrf2 for degradation and responds appropriately to inducers. (A) Chemical structures of inducers. (**B**) HEK293 cells were transfected with the indicated combination of plasmids. 24 h post transfection, the cells were treated with either 0.1 μM CDDO, 10 μM STCA, 5 μM SFN, 400 μM $H_2O_2$ or 10 μM MG132 for 4 h. The level of EGFP–Nrf2 stabilization in response to each inducer was determined by western blot. **(C)** FRAP data.

### 2.5. Transfection

Tubes "A" and "B" each received 100 μl of Opti-MEM (Invitrogen). Plasmid DNA (0.5 μg) was added to "A", except for Nrf2–mCherry for which 0.75 μg of plasmid DNA was used. "B" received 3.75 μl of Lipofectamine 2000 (Invitrogen). Both tubes were incubated for 12 min at room temperature, their contents were mixed, incubated for a further 15 min, and added to cells. After 4.5 h, the Opti-MEM was replaced with complete media.

### 2.6. Microscopy

Cells were washed with PBS, and imaged in 2 ml phenol red free $CO_2$-independent DMEM, supplemented with 10% heat-inactivated fetal bovine serum. For experiments with inducers, 1 ml of media was removed from the dish, and transferred to an Eppendorf tube. To this, 2 μl of a 1000× concentrated solution of inducer was added. After mixing thoroughly, the media was added back to the cells. Time-lapse experiments were carried out using a DeltaVision Spectris wide-field fluorescence microscope with a 60× oil immersion NA 1.4 Plan-Apochromat objective (Olympus), using SoftWoRx software. Images of EGFP and mCherry were taken every 10 min for 5 h. FRAP was carried out using a DeltaVision Spectris wide-field fluorescence microscope with a 60× oil immersion NA 1.4 Plan-Apochromat objective (Olympus), using the photokinetic experiment function of the SoftWoRx software. A small region in the cell was photobleached with a 488 nm laser (100% laser power for 0.1 s), and a time-lapse sequence of EGFP images were taken

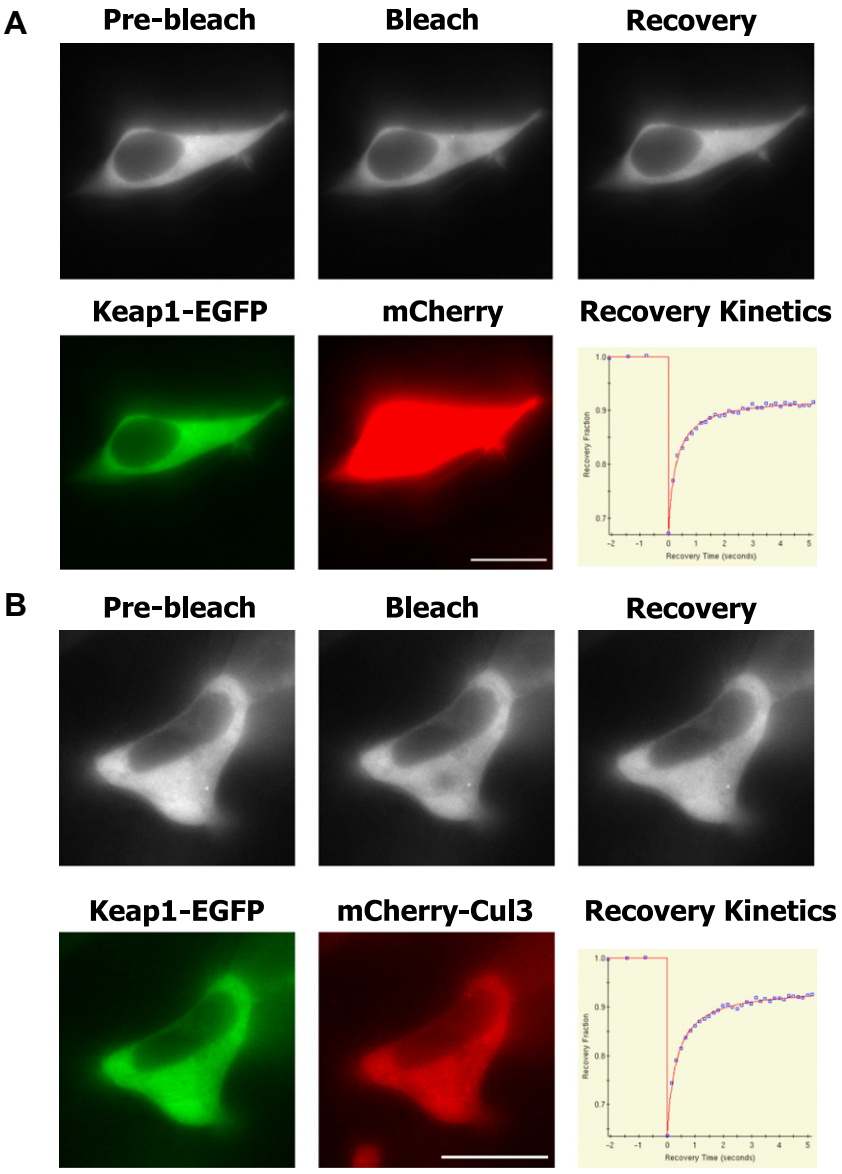



**Fig. 3.** Keap1–EGFP and mCherry–Cul3 interact in live cells. HEK293 cells were transfected with either Keap1–EGFP + mCherry, (**A**) or Keap1–EGFP + mCherry–Cul3, (**B**) and imaged 24 h later. Each cell was imaged three times at basal state (pre-bleach), and 30 times post bleach. The color images show the localization of EGFP and mCherry. The fluorescence recovery dynamics are plotted in the graphs. Scale bar = 15 μm.

(exposure time 0.05 s, bin 2 × 2) to record the fluorescence recovery. Image data were analyzed using SoftWoRx software.

### 2.7. Statistical analysis

Averages, standard deviation and *p*-values were calculated using Excel. The *p*-values were calculated using two-tailed Student's *T*-test.

## 3. Results

### 3.1. A fusion protein of EGFP and Keap1 is functional as a substrate adaptor for mediating Nrf2 degradation and as an inducer sensor

To test whether addition of a fluorescent protein tag to Keap1 will interfere or preserve its function as a substrate adaptor for the ubiquitination of Nrf2, we used a chimeric protein of the green fluorescent protein (EGFP) fused to the N-terminus of Keap1 (EGPF–Keap1), and a fusion protein of Nrf2 with mCherry. As Nrf2 binds to Keap1 through its N-terminal Neh2 domain, we added the mCherry tag to the C-terminus of Nrf2. The EGFP–Keap1 and Nrf2–mCherry fusion proteins were co-expressed in HEK293 cells. We then used live cell imaging in a time-lapse experiment in which a single cell was imaged every 10 min for 5 h. At time 0, EGFP–Keap1 is localized in the cytoplasm and Nrf2–mCherry is undetectable (Fig. 1A), indicating that, similarly to the endogenous Keap1, EGFP–Keap1 efficiently targets Nrf2–mCherry for degradation. After the addition of the potent Michael-acceptor bearing inducer, the oleanane triterpenoid CDDO (Fig. 2**A**), Nrf2–mCherry becomes stabilized as revealed by the time-dependent increase in red fluorescence. Importantly, after treatment with CDDO, Nrf2–mCherry fluorescence was detected in both the cytoplasm and the nucleus, showing that the stabilization of Nrf2–mCherry is followed by its nuclear translocation. This experiment demonstrated that EGFP–Keap1 is functional both as a substrate adaptor for mediating Nrf2 degradation as well as an inducer sensor. Similarly, the addition of the fluorophore mCherry to the C-terminus of

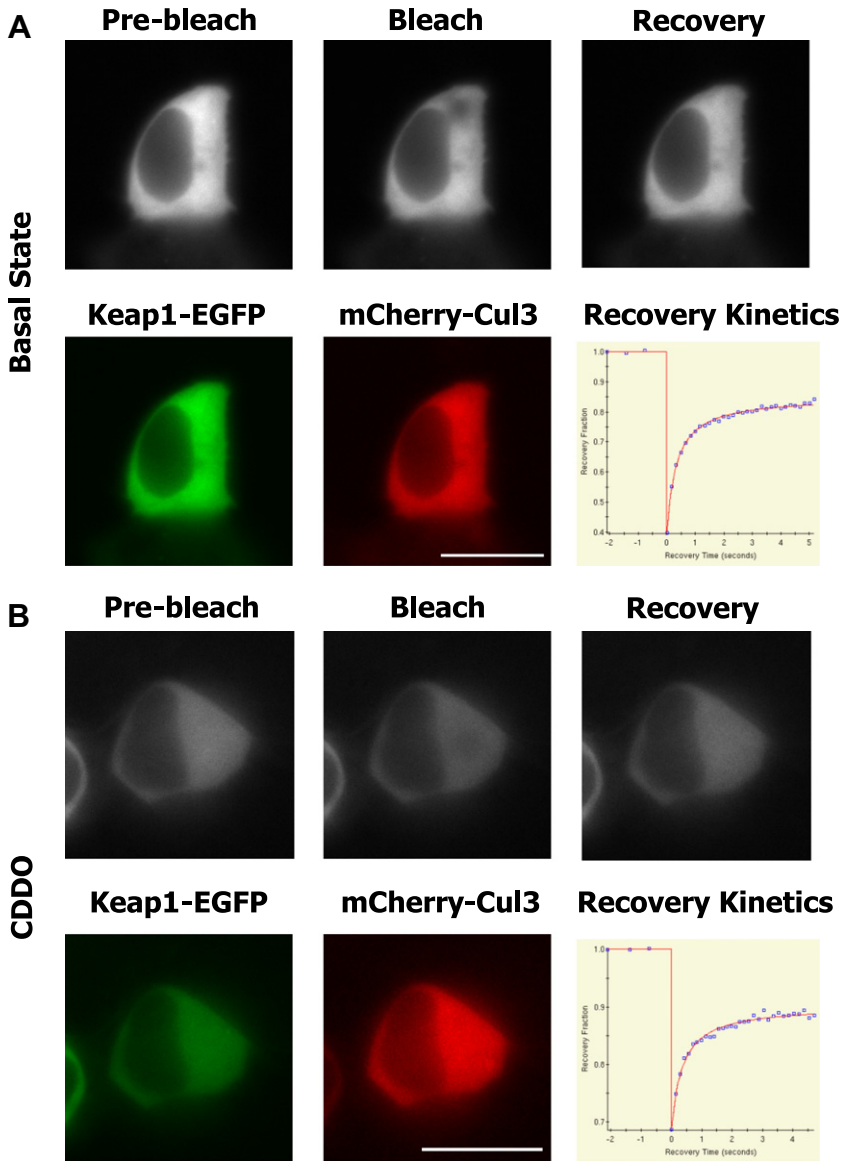



**Fig. 4.** Inducers do not dissociate the Keap1–EGFP:mCherry–Cul3 complex. HEK293 cells were transfected with Keap1–EGFP + mCherry–Cul3, and imaged 24 h later. The images show representative examples of cells in the basal state, (**A**) or after 1-h exposure to 0.1 μM CDDO, (**B**) 5 μM SFN, (**C**) 10 μM STCA, (**D**) or 0.5-h exposure to 400 μM $H_2O_2$. (**E**) Scale bar = 15 μm.

Keap1 did not interfere with the function of Keap1, as it was able to both target EGFP–Nrf2 for degradation in the basal state, and respond to the inducers CDDO, SFN, STCA and $H_2O_2$ in the same way as the endogenous protein (Fig. 2B). The ability of CDDO, and two other inducers, SFN and STCA, to activate Nrf2 was further confirmed in an independent experiment which showed that these inducers caused a dose-dependent induction of the Nrf2-target gene, NAD(P)H:quinone oxidoreductase 1 (NQO1) (Fig. 1**B**).

### *3.2. The fusion proteins Keap1–EGFP and mCherry–Cul3 form a complex in cells*

Cul3-based E3 ubiquitin ligases associate with BTB-domain containing adaptor proteins. As the BTB domain of Keap1 is located at the N-terminus of the protein, we made a second chimeric protein of Keap1 in which EGFP was fused to the C-terminus of Keap1. We then fused mCherry to the N-terminus of Cul3. To test whether the two fusion proteins form a complex, we first co-transfected HEK293 cells with expression plasmids encoding Keap1–EGFP and either free mCherry or mCherry–Cul3, and examined their sub-cellular localization. Similar to endogenous Keap1, the Keap1–EGFP fusion protein localizes predominantly in the cytoplasm, whereas free mCherry is distributed throughout the cell (Fig. 3**A**). The fusion of Cul3 to mCherry changed the localization of the fluorophore from whole cell to cytoplasmic (Fig. 3**B**), suggesting that a Keap1–EGFP:mCherry–Cul3 complex had been formed.

To investigate the diffusion dynamics of the Keap1–Cul3 interaction, we used fluorescence recovery after photobleaching (FRAP). This method measures the rate of diffusion of proteins, and allows the determination of dynamic changes within the complex in which a protein is found as conditions within the cell change [23]. A laser was used to photo-bleach a small area in the cell resulting in the loss or reduction of fluorescence in the bleached region. Due to the natural diffusion of the proteins within the cell, this bleached region recovers its fluorescence as the proteins from the surrounding regions diffuse into the bleached spot. To compare FRAP recovery times between cells, the *T½* (the amount of time re-

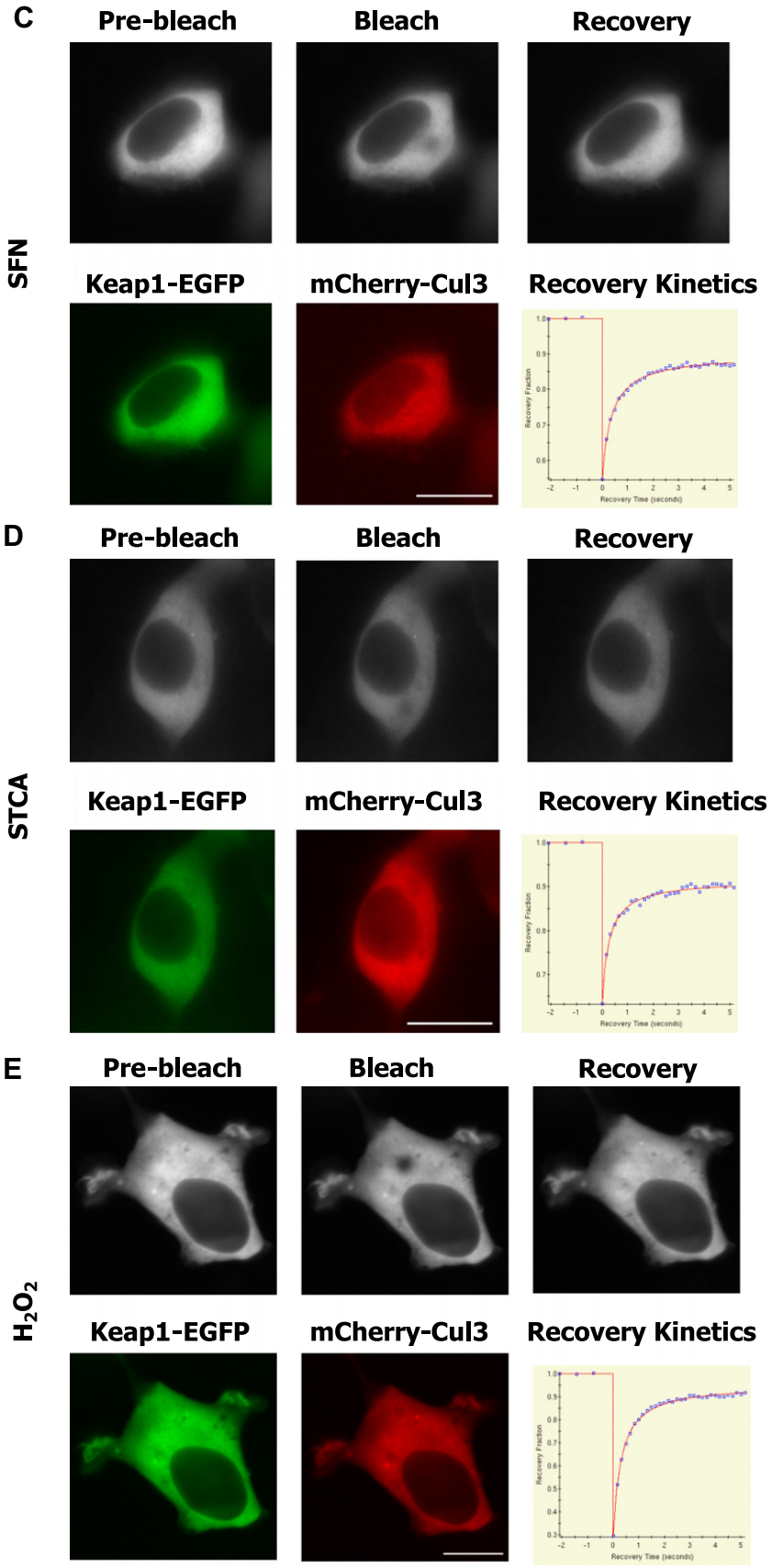



**Fig. 4.** (*continued*)

quired for the fluorescence intensity to reach half of its maximum level during the recovery phase) was calculated for each individual cell. In the absence of Cul3, the *T½* value of Keap1–EGFP was 0.29 s, and when mCherry–Cul3 was co-transfected, the *T½* was increased

to 0.35 s (Fig. 2**C**). This difference was statistically significant ($p = 0.022$) demonstrating that, when co-expressed, Keap1–EGFP and mCherry–Cul3 do bind to one another in live cells.

### 3.3. Inducers do not dissociate the Keap1–EGFP:mCherry–Cul3 complex

We then took advantage of the fact that, if a protein of interest is found within a complex, FRAP allows for the evaluation of the status of the complex as the cellular conditions change. Thus, if introduction of a small-molecule inducer leads to the dissociation of the protein complex, this can be quantified by FRAP as the individual members of the dissociated complex will diffuse faster in the presence of the compound. To determine the effect of inducers on the diffusion dynamics of the Keap1–EGFP:mCherry–Cul3 complex, we first used the triterpenoid CDDO. If the inducer dissociates the Keap1-dependent E3-ubiquitin ligase, then we would expect to see a decrease in the *T½* value after inducer treatment. Firstly, we imaged the cells in the basal state, then added CDDO to the cell culture medium, incubated the cells for 1 h, and imaged the same dish again. Addition of CDDO did not affect the *T½* value (Figs. 4A,B and 2**C**), suggesting that this inducer does not dissociate the Keap1-dependent E3-ubiquitin ligase after a 1-h treatment.

Keap1 is equipped with highly reactive cysteine residues which serve as distinct sensors for specific inducers [15,24,25]. We have previously shown that the triterpenoid TP-225, a closely related analogue of CDDO, reacts with cysteine residues of recombinant Keap1, and that Michael acceptor-bearing triterpenoids, including TP-225 and CDDO, potently induce Nrf2-target genes [19]. Recently, using an Neh2-luciferase reporter it was demonstrated that the methylamide derivative of CDDO activates the reporter dose-dependently within minutes of exposure, and molecular docking analysis has suggested that CDDO-methylamide targets Cys226 of Keap1 [18]. To test whether dissociation of Keap1 from Cul3 may occur by inducers which target other cysteine residues of Keap1, we next used the isothiocyanate SFN which targets Cys151 [14]. As there is currently no crystal structure available of either the full-length Keap1 protein, or the Cul3-BTB domain interface, it is difficult so say with any certainty where the Cys151 residue is located. However, based on the published structures of the BTB-domain protein PLZF and the Cul1 interacting protein Skp1, it has been suggested that β-strand-3 and α-helix-5 within the BTB domain are both important for BTB-Cul3 interactions [26]. Interestingly, Cys151 is located either within or adjacent to α-helix-5, which suggests that, based on its location, modification of Cys151 may disrupt Keap1–Cul3 binding. Similar to CDDO, addition of the Cys151-targeting inducer SFN did not affect the recovery time after photobleaching, and the *T½* value was 0.320 and 0.334 in the vehicle- and SFN-treated cells, respectively (Figs. 4C and 2C). The recovery time was also unaffected when we used the sulfoxythiocarbamate STCA which targets Cys273, Cys288, and Cys613 of Keap1 [17] (Figs. 4D and 2**C**). Finally, addition of the oxidant $H_2O_2$ which promotes formation of a disulfide bridge linking two Keap1 molecules via Cys151 [20] did not lead to a significant change in *T½* either (Figs. 4E and 2C). Thus, exposure of cells for 1 h to inducers of four types with vastly different inducer potencies and targeting distinct cysteine sensors of Keap1 does not dissociate the Keap1–Cul3 complex prior to Nrf2 stabilization and induction of Nrf2-dependent gene expression.

## 4. Discussion

We developed a quantitative FRAP-based system using Keap1–EGFP and mCherry–Cul3 fusion proteins to visualize and investigate the dynamics of the Keap1–Cul3 interaction in single live cells. Structurally-diverse inducers which target distinct cysteine sensors of Keap1 and have vastly different potencies were tested for their ability to dissociate the Keap1–Cul3 complex. Because we wished to determine whether Nrf2 stabilization may be a consequence of the dissociation of Keap1 from Cul3, we chose the time point of 1 h after which to study the effects of inducers. We found that none of the inducers tested changed the *T½* value of Keap1–EGFP. Therefore, we conclude from the FRAP experiments that none of the 4 inducers tested lead to the dissociation of Keap1 from Cul3 after 1 h of treatment.

The previously published data which formed the basis for the model of Keap1–Cul3 dissociation leading to the Nrf2 stabilization is based on co-immunoprecipitation experiments, where inducers were found to cause a modest reduction in the intensity of the pulled-down protein when either Keap1 or Cul3 were used as the bait. Importantly, these experiments have shown reduced binding between Keap1 and Cul3 at later time points than the time point used in our experiments, namely 2 h for oxidized n-3 fatty acids and IAB, and 5 h for tBHQ and SFN. In contrast, inducers stabilize Nrf2 within 30 min of exposure [27,28] and can activate transcription within 1 h [29]. Thus, it is possible that although inducer-mediated dissociation of the Keap1–Cul3 complex can occur at later time points, it is not required for the initial phase of Nrf2 stabilization.

Experiments utilizing the intrinsic tryptophan fluorescence of recombinant Keap1 have revealed that the fluorescence intensity of the native protein is much higher than the fluorescence intensities of either denatured Keap1, an equimolar amount of tryptophan, or a mixture of all amino acids that comprise Keap1 at their respective molar ratios [30]. In addition, inducer binding causes a decrease in intensity and a red shift of the emission spectrum of the Keap1, indicative of a conformational change, which is further supported by the use of a hydrophobicity probe [30]. We therefore conclude that, during the initial phase of Nrf2 stabilization, inducers do not dissociate the Keap1-dependent E3 ubiquitin ligase, but inactivate the substrate adaptor function of Keap1 by causing a conformational change which compromises the correct alignment of the lysine residues within the Neh2 domain of Nrf2 so that ubiquitination does not take place.

## Acknowledgments

We thank John D. Hayes and Michael McMahon (University of Dundee) for kind gifts of plasmids, Young-Hoon Ahn and Philip A. Cole (Johns Hopkins University) for STCA, Michael B. Sporn and Tadashi Honda (Dartmouth College) for CDDO, and David Llères and Samuel Swift (University of Dundee) for help with microscopy. We acknowledge the financial support of the Medical Research Council, Cancer Research UK (C20953/A10270) and Research Councils UK.

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