Evidence that Nek1 does not phosphorylate Rad54-S572 during recovery from IR

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ABSTRACT

A main focus of the work in our lab is on the activity of Tousled Like Kinase 1 (TLK1) in the area of DNA Damage and Repair. As one of its key interactor, TLK1 phosphorylates NIMA related kinase 1 (Nek1), and Nek1 was reported by Spies et al. to phosphorylate and regulate the activity of the key HRR protein Rad54[1], which suggested an intriguing signal transduction pathway: TLK1>Nek1>Rad54. In an effort to confirm such relations, we now report that we have not been able to reproduce key findings from that study. Specifically, we found that Nek1 does not phosphorylate RAD54-S572 as was reported. We generated Nek1-KO mouse NT1 cells[2] and Nek1-Knock-down in Hek293 (same cells as in Spies et al.), and the pRAD54-S572 signal does not change with our custom Ab, with or w/o IR. When we used an Ab from Lobrich's lab, it detected an immunoreactive band of wrong size for RAD54, which also did not change after IR even in synchronized G2 cells, contrary to their report. We also note that their P-assignment was based on guessing a weak consensus Nek1 sequence, and that site-directed mutagenesis of RAD54-S572 failed to yield biological effects in their in in vitro studies[1]. We also could not reproduce their copurification of Nek1-RAD54 by coIP, calling into question this interaction.

INTRODUCTION

Each day our cells face many exogenous and endogenous challenges that threat the genomic stability. The DNA double strand break is the most lethal form of damage. The major mechanism to maintain the fidelity of genetic code is Homologous recombination repair (HRR). The central enzyme of this pathway is Rad54.[3] Rad54 is a well conserved protein of the Rad52 group, a generally well conserved group in eukaryotes. Rad54 is a motor protein that translocates along dsDNA in an ATP hydrolysis dependent manner.[4] In mammals, the RAD54 gene plays its most important role during early developmental stages.[5] The primary function of homologous recombination in mitotic cells is to repair double-strand breaks (DSBs) that form as a result of replication fork collapse, from processing of spontaneous damage, and from exposure to DNA-damaging agents like topoisomerase poisons.[6] Ideally known as the swiss-army knife of homologous recombination repair, Rad54 functions in different stages of HRR. Rad54 interacts with Rad51 and promotes DNA strand -exchange, translocates along donor template, remodels donor chromatin, promotes Branch-migration of Holliday Junctions.[7-10] With such multi-faceted role in vivo, any post-translational modification of Rad54 may alter its activity. Therefore, it is critical to understand the regulation of this error-free mode of DNA repair in cells.

RESULTS

Given the possibility of a TLK1>Nek1>Rad54 axis and its importance for HRR, we wanted to probe the pattern of pRad54-S572 in cells with perturbed TLK1 activity. In an initial communication with Marcus Lobrich, he stated that they had finished the original antiserum published in the Mol Cell paper but that they would ship us a replacement that was not well-characterized. We tested that antiserum during a time-course of recovery from IR of HEK293 cells, either control or expressing a dominant Nek1 kinase-hypoactive mutant (HEK N5)[11], which was expected to show reduced pRad54-S572 signal (Fig.1); and it was immediately obvious that there were problems. The main immunoreactive band ran on the gel faster than its expected position of ~80kDa for Rad54 (Fig1). The signal was not very clean as there were several other (presumably less specific) prominent bands. Most importantly, the intensity of the pRad54 did not increase after recovery from IR, as it was reported. Given the impossibility of reproducing that part of the work with the Ab they provided, we commissioned our own custom-made pRad54-S572 antiserum from Thermofisher. After the 3d boost, an ELISA showed that the serum had a high titer (>$2X10^5$ dilution) and an affinity >100-fold for the phosphopeptide compared to unphosphorylated, even before affinity purification. To establish the specificity of the Ab and its dependence on Nek1, we carried out a siRNA-mediated knockdown of Nek1 in Hek293 cells, similarly to their Fig. 3D

experiment. Despite the successful knock-down of Nek1 (Fig.2), we did not observe any change in the signal with our pRad54-S572 antiserum, which detected a band at the correct position for Rad54 and overlapping the signal obtained with a commercial (santa-cruz sc-166370) pan-Rad54 antiserum. This clearly indicated that Nek1 is unlikely to be the kinase responsible for phosphorylation of S572. Considering the possibility that there might still be sufficient Nek1 in the KD-cells to phosphorylate S572, we took advantage of a mouse PCa cell line in which we clonally knocked-out Nek1 via CRISPR/CAS9 to rule out such possibility. Surprisingly, there was no immunoreactive band with pRad54-S572 antiserum in either control or the Nek1-KO clone – note that these cells do express Rad54L and Rad54B (RNAseq data communicated by Xiuping Yu. In contrast, there was a strong band in HEK293, equally in control and siRNA-KD cells (Fig. 3). This result suggests that despite the high sequence conservation between mouse and human (Fig. 3B), the pRad54-S572 is not found in mouse, and one should wonder whether such proposed phosphorylation is at all important. To probe this question further, we set out to establish if their report of Rad54-S572 phosphorylation after IR, and particularly in late G2, could be reproduced. We thus, synchronized Hek293 cells following release from G1/S block with HU and irradiated them in early G2 (Fig.4), repeating their experimental conditions. Contrary to what they reported, we found no increase in pRad54-S572 in either asynchronous or cells enriched G2 (Fig.5), for which 8h was reported in their paper as the maximal level of phosphorylation[1]. Finally, we were not able to reproduce the reported interaction between Nek1 and Rad54 by coIP, although we cannot rule out that this could be due to a technical failure. Nonetheless, key findings of their paper could not be reproduced. These include a failure to attribute the phosphorylation of S572 to Nek1, its dependency on IR-induced damage, and in general the significance of such Rad54 modification for HRR.

Figure Legends

Figure 1. 2X 10^6 HEK293 cells were seeded 24hrs prior to treatment and then cells were treated with 10Gy of I.R and allowed to recover for indicated times (hrs). 40µg of untreated control (C) and samples were loaded in 8% SDS-PAGE gel and probed with aliquot of antibody sample from Lobrich lab. Tubulin probed as loading control.

Figure 2. 0.3X10^6 HEK293 cells seeded in 6 well plate 24hrs prior to siRNA treatment. Lipofectamine 3000 was used as transfection reagent with indicated amount of siNek1. 40ug of total samples loaded in 8% SDS-PAGE gel and probed with indicated antibodies (Nek1- sc 398813; Rad54- sc 166370; pRad54-Custom generated-Thermofisher; GAPDH- CST #2118)

Figure 3. A) 40µg of cell lysate from NEK1 WT (Cnt), K.O #6 clone (in NT1 cells) and siRNA treated HEK293 cells and control (Cnt) loaded in 8% SDS-PAGE gel and probed for Nek1 and pRad54 (custom generated) antibody. Actin band shown as loading control. B) Human Rad54 and mouse Rad54 protein sequence alignment shown for the conservation of the S572 site.

Figure 4. Cell cycle analysis of HEK293 cells synchronised (asynchronous -Asyn) at G1/S with Hydroxyurea treatment and released for indicated times (as shown in schematic).

Figure 5. Synchronised and aynchronised HEK293 cells were untreated or irradiated (5 Gy) at G2 and allowed to recover for indicated times. 40ug of cell lysate was loaded in 8% SDS-PAGE gel and probed with indicated antibodies. Ku-70 probed for loading control.

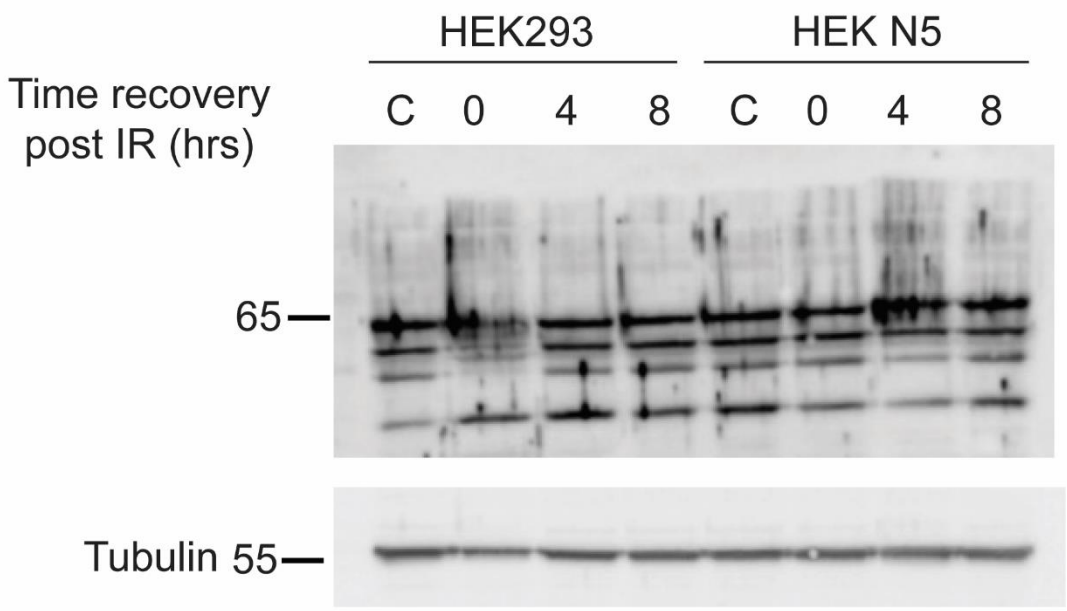
HEK293
HEK N5
Time recovery post IR (hrs)
C 0 4 8 C 0 4 8
65
Tubulin 55

Fig. 1

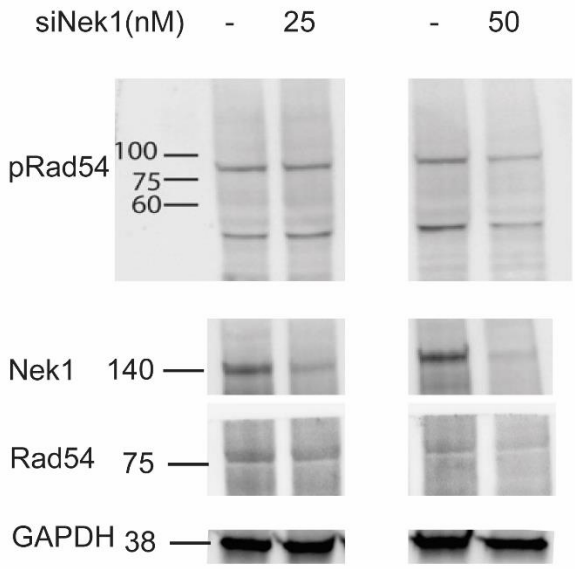
siNek1(nM) - 25 - 50
pRad54
100
75
60
Nek1 140
Rad54 75
GAPDH 38

Fig. 2

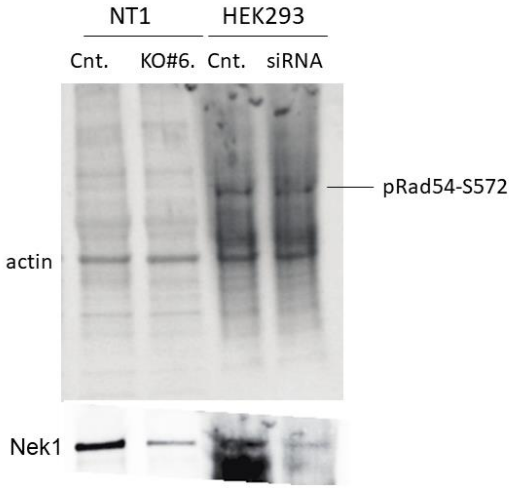

Fig.3A

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hRad54 VRLDGTMSIKKRAKVVERFNSPSSPDFVFMLSSKAGGCGLNLIGANRLVMFDPDWNPAND 600
mRad54 VRLDGTMSIKKRAKVVERFNSPSSPDFVFMLSSKAGGCGLNLIGANRLVMFDPDWNPAND 600
```

Fig.3B

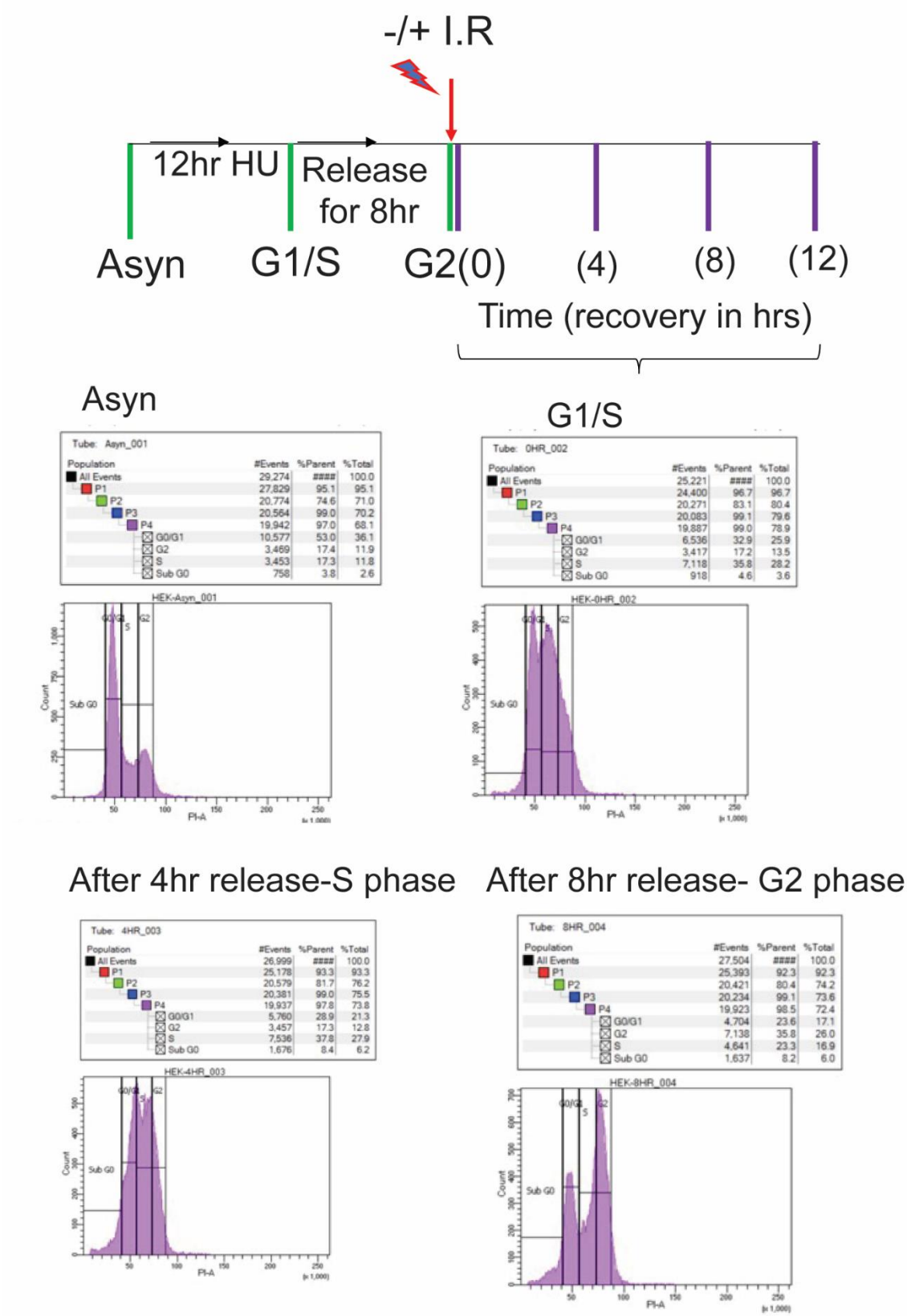
-/+ I.R
12hr HU
Release for 8hr
Asyn
G1/S
G2(0)
(4)
(8)
(12)
Time (recovery in hrs)
Asyn
Tube: Asyn_001
Population #Events %Parent %Total
All Events 29,274 #### 100.0
P1 27,829 95.1 95.1
P2 20,774 74.6 71.0
P3 20,564 99.0 70.2
P4 19,942 97.0 68.1
G0/G1 10,577 53.0 36.1
G2 3,469 17.4 11.9
S 3,453 17.3 11.8
Sub G0 758 3.8 2.6
HEK-Asyn_001
G0/G1
S
G2
Sub G0
Count
PI-A
G1/S
Tube: 0HR_002
Population #Events %Parent %Total
All Events 25,221 #### 100.0
P1 24,400 96.7 96.7
P2 20,271 83.1 80.4
P3 20,083 99.1 79.6
P4 19,887 99.0 78.9
G0/G1 6,536 32.9 25.9
G2 3,417 17.2 13.5
S 7,118 35.8 28.2
Sub G0 918 4.6 3.6
HEK-0HR_002
After 4hr release-S phase
Tube: 4HR_003
Population #Events %Parent %Total
All Events 26,999 #### 100.0
P1 25,178 93.3 93.3
P2 20,579 81.7 76.2
P3 20,381 99.0 75.5
P4 19,937 97.8 73.8
G0/G1 5,760 28.9 21.3
G2 3,457 17.3 12.8
S 7,536 37.8 27.9
Sub G0 1,676 8.4 6.2
HEK-4HR_003
After 8hr release- G2 phase
Tube: 8HR_004
Population #Events %Parent %Total
All Events 27,504 #### 100.0
P1 25,393 92.3 92.3
P2 20,421 80.4 74.2
P3 20,234 99.1 73.6
P4 19,923 98.5 72.4
G0/G1 4,704 23.6 17.1
G2 7,138 35.8 26.0
S 4,641 23.3 16.9
Sub G0 1,637 8.2 6.0
HEK-8HR_004

Fig.4

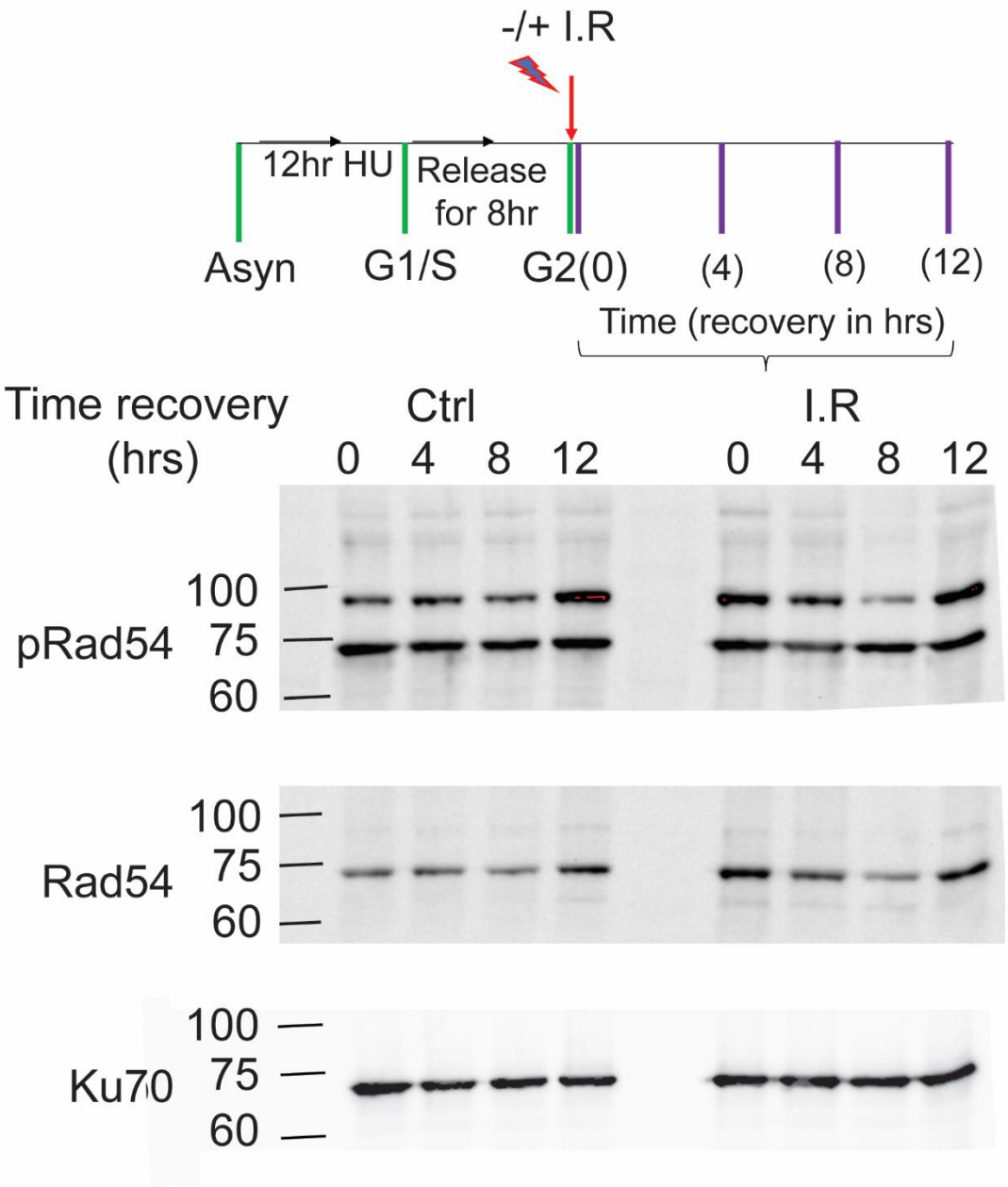
-/+ I.R
12hr HU
Release
for 8hr
Asyn
G1/S
G2(0)
(4)
(8)
(12)
Time (recovery in hrs)
Time recovery
(hrs)
Ctrl
I.R
0 4 8 12
0 4 8 12
100
75
60
pRad54
100
75
60
Rad54
100
75
60
Ku70

Fig. 5

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