Reactive oxygen species in health and disease
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The effect of mitochondrial ROS on nuclear epigenetics

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Abstract
Mitochondria generate reactive oxygen species (ROS) as a byproduct of the oxidative phosphorylation. Short term increases in mitochondrial ROS (mtROS) production boost the intracellular ROS protection mechanisms, even for a long time after this initial mtROS exposure. This enables the cell to be better protected while being exposed to another increase in ROS later on in life. This response may delay or even prevent diseases, and thus, can contribute to longevity. We hypothesized that mtROS molecules can signal to the nucleus and can change the epigenetic landscape in such a way that this ROS protective response is being stored for the long term. Possibly, the effect of mtROS is dependent on the specific location in the mitochondria where it is produced. Therefore, to study the role of mtROS on nuclear epigenetics, we generated three differentially-targeted (mitochondrial DNA, matrix and intermembrane space) SuperNova fluorescent proteins. These SuperNova fusion-proteins can generate ROS upon light exposure of a certain wavelength, and as such, can be used to determine the effect of mtROS on genome-wide and locus-specific epigenetic changes in the nucleus. Such research is needed to better understand how mitochondria communicate with the nucleus, and as such provide new insights into the role of mitochondria in health and disease.
Introduction
Mitochondria originate from bacteria that entered a eukaryotic cell and formed a symbiotic relationship (1). During many years of evolution most (>99%) of the mitochondrial proteins are transferred from the mitochondrial to the nuclear DNA. As a result, mitochondrial function is highly dependent on transcription/translation of mitochondrial proteins encoded in the nuclear DNA. Therefore, a good communication between both organelles is essential for the regulation of mitochondrial biogenesis. Moreover, this communication is essential for the mitochondria to adequately respond to stress, e.g. oxidative stress.

A variety of different signaling molecules can facilitate this mitochondria-to-nucleus signaling (also called mitochondrial retrograde signaling) (as reviewed in (2)). One example of the messenger molecules important for this communication are the reactive oxygen species (ROS), more specifically the superoxide anion (O$_2^•$−) and hydrogen peroxide (H$_2$O$_2$). Mitochondria are one of the main producers of these ROS molecules (3); during the oxidative phosphorylation (OXPHOS), electrons are pumped over the mitochondrial membranes in order to produce ATP, the main fuel of the cell. A small percentage of these electrons leak out of the electron transport chain and can react with oxygen to form O$_2^−$. O$_2^−$ can be converted to H$_2$O$_2$ either spontaneously or catalytically by superoxide dismutase (SOD). O$_2^•$− is generated either in the mitochondrial matrix or intermembrane space. While O$_2^•$− can cross the outer mitochondrial membrane only via voltage-dependent anion-selective channels (VDACs), H$_2$O$_2$ can freely diffuse across the mitochondrial membranes (4) (Figure 1). This, in combination with its relative low reactivity and long half-life, makes H$_2$O$_2$ an excellent second messenger (5); the half-life of H$_2$O$_2$ in lymphocytes is 1 ms while that of O$_2^•$− is 1 µs (6).

Depending on the level, ROS can be either detrimental or beneficial; high levels of mitochondrial ROS (mtROS) can induce damage and accelerate aging, whereas low levels can improve long-term stress resistance by inducing an adaptive response, which has been termed mitohormesis (7). This coordinated transcriptional response in the nucleus that is triggered by low-dose mtROS, promotes health by preventing or delaying a number of chronic diseases, and ultimately extends lifespan (8-10).

In worms it was found that, in contrast to mitochondrial O$_2^•$−, cytoplasmic O$_2^•$− does not increase, but even decrease, the lifespan (11). This indicates that the ROS affect an unknown factor specifically in the mitochondria that transfers the “mitohormetic/longevity” message to the nucleus. Many factors and signaling
pathways can induce such a low-dose mtROS mediated communication to the nucleus, among which calorie restriction, hypoxia, temperature stress, physical activity, and downstream signaling of insulin/IGF-1 receptors, AMP-dependent kinase (AMPK), target-of-rapamycin (TOR), and sirtuins (12).

Not only studies in lower organisms or animal models have provided evidence for the existence of a mitohormetic response (7); in humans, it has been shown that antioxidant usage during physical exercise blocks the beneficial effects of exercise, including the induction of insulin sensitivity and the induction of antioxidant defense genes (13). This suggests that the ROS produced during physical activity are the origin of, and are required for, some of the long-term health-promoting effects associated with exercise.

**Figure 1. ROS production by the complexes of the mitochondrial oxidative phosphorylation.** During the oxidative phosphorylation (OXPHOS), mitochondria pump electrons over the inner mitochondrial membrane, resulting in the production of ATP. In eukaryotic cells, five protein complexes are involved in this process: complex I-V. Three of these complexes (I-III) are producing the ROS molecule $\text{O}_2^-$ as a side-product. $\text{O}_2^-$ can be produced both in the mitochondrial matrix (complex I-III) and the intermembrane space (complex III). The antioxidant superoxide dismutase (SOD) can convert $\text{O}_2^-$ to $\text{H}_2\text{O}_2$ (SOD1 in the intermembrane space, and SOD2 in the matrix), and this ROS can be neutralized to $\text{H}_2\text{O}$ and $\text{O}_2$ by enzymes such as glutathione peroxidase (GPX). Despite this, not all ROS are neutralized. Some ROS may diffuse out of the mitochondria and contribute to mitochondria-to-nucleus signaling. $\text{H}_2\text{O}_2$ can both freely diffuse across the mitochondrial membranes, and can be transported across the inner mitochondrial membrane via aquaporin-8 (AQP8). In contrast, $\text{O}_2^-$ cannot freely diffuse across the mitochondrial membranes. However, when produced in the intermembrane space, $\text{O}_2^-$ can cross the outer mitochondrial membrane via a voltage-dependent anion-selective channel (VDAC).
As cells “memorize” previous oxidative insults in the mitochondria (as discussed above), we hypothesize there is an epigenetic basis for this mitohormetic response. mtROS might be the component that defines this epigenetic basis by influencing nuclear epigenetics; ROS might directly modify the DNA/epigenetic enzymes (14) or an indirect action of ROS, acting via the induction of mitochondrial dysfunction, might change epigenetic enzyme activity via a change in metabolism, and as such epigenetic enzyme co-factor availability (15, 16) (Figure 2).

**Figure 2. Krebs/citric acid cycle.** The citric acid cycle occurs in the matrix of the mitochondria. Many metabolites (encircled in red) that are used in this metabolic pathway also function as co-factor for the activity of epigenetic enzymes.

It is unclear which ROS molecules may contribute to the mitohormetic response and how they might affect nuclear epigenetics. As mentioned before, H$_2$O$_2$ seems to have the most favorable characteristics (4-6) to act as signaling molecule in the mitohormetic response. Nevertheless, some evidence in budding yeast cells (*Saccharomyces cerevisiae*) also points toward an important role of O$_2^{•−}$ in this response; when yeast cells are exposed to H$_2$O$_2$, they will protect themselves against this stressor by inducing mitochondrial O$_2^{•−}$ production produced by complex III (17). This response is independent of the conversion of O$_2^{•−}$ to H$_2$O$_2$, as mutant yeast unable to produce mitochondrial O$_2^{•−}$ are vulnerable toward H$_2$O$_2$. As O$_2^{•−}$ cannot diffuse across the inner mitochondrial membrane, the location of mitochondrial O$_2^{•−}$ production (mitochondrial matrix versus intermembrane space) may also have a major influence on its role in the mitohormetic response. Matrix produced O$_2^{•−}$ has to be converted to H$_2$O$_2$ before it may signal to the nucleus. Moreover, depending on the location, the cytotoxic
threshold of ROS may be different, as the antioxidant capacity might be different, or ROS-induced damage to specific mitochondrial components (e.g. mitochondrial DNA is localized in the matrix) may be more cytotoxic than others.

As it is unclear whether and how the location of mtROS production may affect its effect on nuclear epigenetics, three differentially-targeted (mitochondrial DNA, matrix and intermembrane space) SuperNova proteins were generated. Upon exposure to light of a specific wavelength, the photosensitizing fluorescent protein SuperNova will generate \( \cdot O_2^- \) and \( O_2^1 \). Using this system, we will study the effect of sub-cytotoxic ROS levels in these different compartments of the mitochondria towards nuclear DNA methylation and histone modifications.

**Results**

**Mitochondrial localization of differentially-targeted SuperNova proteins**

To investigate the effect of location of mtROS production on nuclear epigenetics, three differentially-targeted (mitochondrial DNA, matrix and intermembrane space) SuperNova proteins were cloned. Initial testing of proper localization of the constructs was performed by transiently transfecting HEK293T cells with these constructs. The first construct that was generated, pCDH-\( \text{mtZFP-\text{SuperNova}} \) contained one mitochondrial localization (MLS), a zinc finger protein (ZFP), and one nuclear export signal (NES). The ZFP is a DNA binding protein that was designed in such a way that it binds to a specific 9bp region in the mtDNA. The NES was included to overcome the nuclear localization signal that is inherent in the ZFP. To determine mitochondrial localization of this construct, mitochondria of transfected HEK293T cells were stained with Mitotracker (**Fig. 3**). As seen in **Fig. 3**, confocal microscopy images of pCDH-\( \text{mtZFP-\text{SuperNova}} \) (1x MLS, 1x NES) show nuclear localization of this construct.

To overcome the nuclear localization, an additional MLS and NES were cloned into pCDH-\( \text{mtZFP-\text{SuperNova}} \). This construct was also transfected in HEK293T cells and mitochondria were stained by Mitotracker. The additional MLS and NES resulted in predominant (~90%) mitochondrial localization (**Fig. 4**). Based on this finding, it was decided to include two MLSs and NESs in each of the differentially-targeted SuperNova and mCherry constructs. mCherry constructs were used as a negative control, as this fluorescent protein has similar fluorescent properties as SuperNova but does not produce ROS upon excitation (18).
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**mtZFP-SuperNova** | **Mitotracker Deep Red** | **Overlay** | **DIC**
---|---|---|---

**Figure 3. mtZFP-SuperNova (1x MLS, 1x NES) localizes to the nucleus.** HEK293T cells were transfected with pCDH-mtZFP-SuperNova. 72h post transfection, mitochondria were stained for 30 min with 100 nM Mitotracker Deep Red.

**mtZFP-SuperNova** | **Mitotracker Deep Red** | **Overlay** | **DIC**
---|---|---|---

**Figure 4. mtZFP-SuperNova (2x MLS, 2x NES) localizes predominantly to the mitochondria.** HEK293T cells were transfected with pCDH-mtZFP-SuperNova. 72h post transfection, mitochondria were stained for 30 min with 100 nM Mitotracker Deep Red.

Subsequently, HeLa cells were lentivirally transduced with pCDH-mtZFP-SuperNova or pCDH-mtZFP-mCherry (2x MLS, 2x NES). After puromycin selection, localization of the construct was determined by confocal microscopy. Stable expression of both constructs resulted in mitochondrial localization (**Fig. 5**).

**Binding of mtZFP to its target region in the mitochondrial DNA**

To determine whether mtZFP binds to its target region in the mitochondrial DNA, a chromatin immunoprecipitation (ChIP) was performed. As shown in **Fig. 6**, binding of mtZFP to its target region was significantly enriched (HAtag: 13.2 ± 1.2% of input) compared to the same construct without mtZFP (HAtag: 2.35 ± 0.23% of input).
Figure 5. **mtZFP-SuperNova (A) and mtZFP-mCherry (B)** (2x MLS, 2x NES) localize to the mitochondria. HeLa cells were stably transduced with pCDH-mtZFP-SuperNova (A) or pCDH-mtZFP-mCherry (B). Mitochondria were stained for 30 min with 100 nM Mitotracker Deep Red.

Figure 6. **mtZFP binds to its designed target region.** mtZFP binding (HA-tag) to its designed target region was validated by quantitative ChIP, IgG was used as negative control. Each bar represents the mean ± SEM. Two-tailed t-test, *p < 0.05.

**Mitochondrial ROS production**

After determining correct localization (**Fig. 5-6**) of pCDH-mtZFP-SuperNova and pCDH-mtZFP-mCherry in HeLa cells, mitochondrial $O_2^{-.}$ production was determined using the MitoSOX ROS probe. To activate SuperNova ROS production, HeLa cells stably expressing mtZFP-SuperNova were exposed to light irradiation by different light sources (#1-#3) for different amount of times. HeLa cells stably expressing mtZFP-mCherry were used as a negative control, and to account for aspecific ROS production by these light sources, e.g. heat-induced ROS. As shown in **Fig. 7**, mitochondrial $O_2^{-.}$ production did not significantly
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increase after light irradiation using three different light sources in HeLa cells stably expressing mtZFP-SuperNova compared to mtZFP-mCherry. This result could not be explained by the experimental setup to detect the mitochondrial O$_2^{-}$, as the MitoSOX Red ROS probe functioned as expected using a positive (100 µM menadione for 1h) and negative control (100 µM menadione for 1h with pretreatment using 0.82 mg/ml N-acetyl cysteine (NAC)). Moreover, when exposed to the same amount of light irradiation, no difference in cytotoxicity was observed between the mtZFP-SuperNova compared to the mtZFP-mCherry expressing cells (data not shown). Concluding from this, either the light irradiation was not sufficient or, more likely, the SuperNova protein in the fusion context could not produce ROS anymore.

Figure 7. Mitochondrial ROS production is not increased upon light irradiation of mtZFP-SuperNova. HeLa cells stably expressing mtZFP-SuperNova, or mtZFP-mCherry as negative control, were exposed to light from source #1 (A), #2 (B), or #3 (C). After a certain amount of light exposure, mitochondrial O$_2^{-}$ production was determined with the MitoSOX Red ROS probe. To confirm successful detection of mitochondrial O$_2^{-}$, HeLa cells were exposed to 100 µM menadione for 1h with (- control) or without (+ control) an O/N pretreatment with 0.82 mg/ml NAC.
ROS can change nuclear epigenetics

Although we succeeded in targeting SuperNova to the mitochondria, we could not test the effect of mtROS on nuclear epigenetics in our system. However, as a previous study by Niu et al. suggested that a 3h H₂O₂ treatment in BEAS-2B cells can induce H3K4me3 by 1.5 fold (19), we performed a western blot to confirm their data. Indeed, also in our hands H₂O₂ treatment for 3h increased H3K4me3 by 1.5 fold (Fig. 8).

![Western blot against H3K4me3 and H3 core (loading control) containing protein lysates of untreated (UT) or 3h 250 μM H₂O₂ treated BEAS-2B cells. Below the figure H3K4me3 is quantified compared to the H3 core loading control and normalized to UT cells.](image_url)

**Figure. 8: H₂O₂ treatment induces H3K4me3.** Western blot against H3K4me3 and H3 core (loading control) containing protein lysates of untreated (UT) or 3h 250 μM H₂O₂ treated BEAS-2B cells. Below the figure H3K4me3 is quantified compared to the H3 core loading control and normalized to UT cells.

**Discussion**

To reveal the effect of mtROS on nuclear epigenetics, innovative systems need to be created in which mitochondrial O₂•⁻ can be produced in a time- and space-controlled fashion. Such systems would allow to give us a greater insight at the molecular level in the health-promoting effects of factors such as physical exercise and dietary restriction. These insights are urgently needed to come up with new strategies to prevent diseases associated with our Western life style (sedentary life style, with an abundance of food).

Unfortunately, the mitochondria-targeted SuperNova proteins did not generate mtROS in our experiments. Else we would have looked into the mtROS induced genome-wide epigenetic changes of histone modifications by Western blotting and DNA methylation by LUMinometric Methylation Assay (LUMA) (20) and pyrosequencing of long interspersed nuclear elements (LINE)-1 (21). LUMA is a technique based on a pair of methylation-sensitive/insensitive restriction enzymes. LINE-1 are retrotransposons that compromise about 17% of the human genome (22), and as such these can be used as a surrogate marker for global changes in nuclear DNA methylation levels.

There are some indications from literature, that nuclear epigenetic changes induced by hormetic (mt)ROS levels can be detected at the global level. For example, both global and local hydroxymethylcytosine (hmC) levels were changed in human neuroblastoma cells treated with buthionine sulfoximine (BSO, causing glutathione depletion (major antioxidant), and therefore ROS induction), and in mice depleted for the major antioxidant enzymes glutathione peroxidase 1...
and 2. Interestingly, the hmC level was particularly affected in genes important for protective responses to oxidative stress (23).

Another study has shown that H$_2$O$_2$ can modulate the nuclear epigenetic landscape by reducing activity of the Fe(II) and alpha-ketoglutarate (αKG)-dependent dioxygenases including the ten-eleven translocation (TET) and histone demethylase enzymes (19). In this study, Niu et al. could show that oxidative stress reduces the portion of active enzyme by preventing the reduction of Fe(III) back to Fe(II). This ROS-mediated reduction of the Fe(II) pool might partly explain the differential hmC levels found by Delatte et al. (23). As a result of reduction of the Fe(II) pool, histone methylation and DNA demethylation were reduced; After short term exposure (3h, 250 μM) to H$_2$O$_2$, H3K9Ac and H4K8Ac levels were decreased, whereas H3K4me3 was increased. On the other hand, long term treatment (3 weeks, 25 μM) did not affect histone acetylation, but did increase histone methylation (H3K4me3 > H3K9me3 > H3K27me3). However, these changes in histone modifications, induced by a 3 week H$_2$O$_2$ exposure, were not sustained after a 3 or 6 day washout period.

Interestingly, after 3 weeks of H$_2$O$_2$ treatment, the carbonylated protein content (representing the level of protein oxidation, being a biomarker of oxidative stress), was not significantly increased, in contrast to the 3h treatment. Based on this, long-term oxidative stress may lead to cellular adaptations that help the cell to cope with this stressor. As these long-term oxidative stress induced global changes in histone modifications were not sustained, it could be that only the histone modifications in specific genes (i.e. genes important in the mitohormetic response) are being sustained, and therefore, global histone modification levels are not detectably altered.

As some of the nuclear epigenetic changes caused by hormentic mtROS levels may not be visible at the genome-wide level, it is interesting to interrogate specific genes or specific loci as well. Our initial focus would have been on nuclear genes that are known to be important for the communication between mitochondria and nucleus, such as TFAM, PGC1α, NRF1, OGG1, FOXO1, p53 and genes involved in AMPK and mTOR signaling (24). Moreover, based on a study in budding yeast, subtelomeric regions might also be specifically altered by hormentic mtROS; in these cells, it was observed that hormentic menadione treatment (inducing mtROS, but not DNA damage) could extend yeast chronological lifespan (25). This mtROS signal was sensed by Tel1p and Rad53p, homologs of the mammalian DNA damage response kinases ATM and Chk2. Subsequently, phosphorylation of the H3K36me3 histone demethylase Rph1p, related to
mammalian Jumonji-domain histone demethylases, resulted in increased H3K36me3 levels specifically at subtelomeric regions. As a consequence, binding of the silencing Sir2 (NAD+ dependent histone deacetylase) binding protein Sir3p was enhanced, resulting in repressed transcription from these regions. Whether similar signaling pathways also take place in human cells, still needs to be resolved. Nevertheless, first indications point to conserved pathways between yeast and human; in line with yeast Tel1p, ATM has been found to be a redox sensor in human cells, independent of DNA damage (26).

Based on the literature discussed above, hormetic mtROS signaling likely affects nuclear epigenetics in human cells. However, the exact mechanisms remain largely elusive. Both redox-sensitive second messengers as mtROS itself may signal to the nucleus (27). An interesting observation in rat pulmonary endothelial artery cells, might give us a first clue how sufficient mtROS can reach the nucleus to affect transcriptional regulation; in these cells, hypoxia-induced generation of mtROS has been shown to result in perinuclear mitochondrial clustering. This was necessary to accumulate ROS in the nucleus, and induce hypoxia-induced transcriptional regulation (28). It would be interesting to determine whether the hormetic mtROS signaling is also associated with this perinuclear mitochondrial clustering.

In conclusion, hormetic mtROS signaling may affect both co-factor availability and direct function of epigenetic enzymes. However, future efforts are required to gain insight into the exact mechanisms. The experimental tools described in this study, may, when further optimized, help us to gain this insight.

Materials and methods

Cell culture
BEAS-2B (human bronchial epithelium) HeLa (human cervical cancer) and HEK293T (human embryonic kidney) cells were obtained from the ATCC. BEAS-2B cells were cultured in collagen-coated flasks. Cells were cultured in RPMI 1640 medium (Lonza) (BEAS2-B) or DMEM (HeLa, HEK293T) supplemented with 10% FCS (Perbio Hyclone), 50 μg/mL gentamycin sulfate (Invitrogen), 2 mM L-glutamine (BioWhittaker). All cells were kept at a humidified incubator with 5% CO₂ at 37 °C.
Design and cloning of the differentially-targeted (mitochondrial DNA, matrix and intermembrane space) SuperNova constructs

**Master synthetic construct**
The differentially-targeted SuperNova proteins were all cloned using one master synthetic construct. This construct was synthesized at Bio Basic Canada and contains (from 5’- to 3’-end): 1. Kozak sequence; 2. N-terminal 49-aa mitochondrial localization signal (MLS) of the F1β subunit of mitochondrial ATP synthase (29); 3. engineered three-finger zinc finger protein (ZFP) (see below for more details); 4. HA-tag; 5. 17-aa flexible linker – (SGGGG)3SS (29); 6. SuperNova nucleotide sequence (GenBank AB522905, (18)); 7. C-terminal 18-aa nuclear export signal (NES) of the nonstructural protein 2 of minute virus of mice (30); 8. stopcodon. The addition of restriction enzymes between the individual components enabled flexibility in cloning of the differentially-targeted SuperNova proteins: *BamHI* – Kozak – MLS – *NruI*…*AvrII* – ZFP – *BsiWI*…*NruI* – HA-tag – flexible linker – *EcoRV*…*Ascl* – SuperNova – *Paci*…*EcoRV* – NES – stopcodon – *NotI*. This master construct was subcloned into pCDH-CMV-MCS-EF1-copGFP (CD511B-1) using *BamHI* and *NotI* restriction sites (System Biosciences). In this plasmid EF1-copGFP was swopped with SV40-puromycin resistance using *NotI* and *XhoI* restriction enzymes. As described below, modifications of this construct (pCDH-CMV-master synthetic construct-SV40-puro) enabled targeting of the SuperNova protein to: 1. a specific location in the mtDNA; 2. mitochondrial matrix; 3. mitochondrial intermembrane space.

As a negative control, SuperNova was replaced by mCherry using *Ascl* and *Paci* cloning sites. Like SuperNova, mCherry is a monomeric fluorescent protein with comparable fluorescent spectra. However, in contrast to SuperNova, mCherry is unable to produce ROS upon light irradiation.

**Mitochondrial DNA targeting SuperNova protein (pCDH-mtZFP-SuperNova):**
The online tool [www.zincfungertools.org](http://www.zincfungertools.org) was used to search for a suitable target region for an engineered ZFP consisting of three fingers fused together to target a unique 9 bp mitochondrial DNA region (H-strand: GAG GAG GTA) near the HSP1 and HSP2 promoters (31). An additional MLS (*AvrII* restriction sites) and NES (*EcorV* restriction sites) were inserted into the master synthetic construct, which generated pCDH-mtZFP-SuperNova (Fig. 9) and enabled successful targeting of the ZFP-SuperNova fusion protein to the mitochondrial DNA.
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**Figure 9: Design of pCDH-mtZFP-SuperNova.**

*Mitochondrial matrix targeting SuperNova protein (pCDH-MLS-SuperNova):*  
In order to target the SuperNova protein to the mitochondrial matrix, but not to a specific region of the mtDNA, the ZFP was removed from pCDH-mtZFP-SuperNova using *NruI* digestion. This resulted in pCDH-MLS-SuperNova (Fig. 10).

**Figure 10: Design of pCDH-MLS-SuperNova.**

*Mitochondrial intermembrane space targeting SuperNova protein (pCDH-IMS-SuperNova):*  
In order to target the SuperNova protein to the mitochondrial intermembrane space, the MLS was removed from pCDH-MLS-SuperNova and replaced by the first 61 amino acids of yeast cytochrome C1 containing an IMS (32) using *BamHI* and *BstII* digestion. This resulted in pCDH-IMS-SuperNova (Fig. 11).
Figure 11: Design of pCDH-IMS-SuperNova.

Transfection and transduction
The calcium phosphate method was used to produce lentiviral particles containing one of the differentially-targeted SuperNova proteins. The same method was used to transfect HEK293T cells (without resulting in viral particle production), but for this, the viral packaging plasmids were excluded from the transfection mix. For lentiviral particle production, HEK293T packaging cells were co-transfected with plasmids containing the differentially-targeted SuperNova construct, and viral packaging plasmids containing gag/pol and the vesicular stomatitis virus G protein in a 3:2:1 ratio, as described before (33). Viral supernatant was collected 48h and 72h post transfection and was used in combination with 6 µg/ml polybrene (Sigma-Aldrich) to infect HeLa host cells. Two days post transduction, HeLa cells stably expressing the SuperNova containing plasmid were selected for 5-7 days by 2 µg/ml puromycin.

Confocal microscopy
Correct localization of the cells expressing the differentially-targeted SuperNova or mCherry proteins was determined using confocal fluorescent microscopy (Leica SP2, HCX PL APO 63x/1.4 lens). Mitochondria were stained by treating cells with 100 nM Mitotracker Deep Red FM (Molecular Probes) for 30 min at 37°C. SuperNova and mCherry were excited using a 543 nm and Mitotracker Deep Red was excited using a 633 nm HeNe laser light.
**Chromatin immunoprecipitation (ChIP)**

Binding of the ZFP to its designed target region was determined by ChIP. Cells were fixed with 1% formaldehyde for 8 min at RT. After two PBS washes, cells were lysed and subsequently sonicated for 8 min using a Bioruptor (Diagenode; 4 cycles of 30’’ on, 30’’ off). Sheared chromatin was collected by centrifugation for 10 min at 13,000 rpm at 4 °C. 40 µl magnetic beads (Life technologies) were coated for 10 min at RT with 4 µg antibody (Rabbit IgG, Ab46540; HA-tag, Ab9110). After washing the beads with 0.02% PBS-Tween, sheared chromatin of 25 x 10⁴ cells was added to the precoated magnetic beads and incubated O/N at 4 °C. The following day, unbound chromatin was collected from the IgG control IP. All beads were washed 3 times with PBS and DNA was eluted with elution buffer (1% SDS, 100 mM NaHCO₃). Eluted DNA was RNase (Roche) treated under high salt conditions O/N at 62 °C. The next day, after a 1 h incubation at 62 °C with proteinase K (Thermo Scientific), DNA was purified with the Qiagen Qiaquick PCR Purification kit (Qiagen). qPCR was performed using primers specific for the ZFP target region (FW: 5’-TTACCTCCTCAAAGCAATACACTG-3’, RV: 5’-TGATTTAGAGGTGAACCTGGA-3’) to determine relative enrichment of the HA-tag in this region with the formula: percentage input = 2^(Ctinput – CtChIP) * dilution factor * 100.

**Light irradiation**

Three different light sources were used to irradiate HeLa cells expressing SuperNova or mCherry protein:

#1. 100 x 100 mm, 520±25 nm, LED backlight (Advanced Illumination)
#2. Osram Dulux F 36W/830 (Osram).
#3. Ultrabright lime (567 nm) Rebel 7-LED light system (Luxeon Star LEDs) focused in one small beam with the 12° 39 mm Circular Beam Optic (Polymer Optics).

Light irradiation was performed either inside (#1) or outside (#2 and #3) the incubator. When the set-up did not allow light irradiation inside the incubator, cells were kept warm on a 37°C heat plate (#2). To minimize the thermal damage induced by the heat produced by light source #3, a T75 flask filled with water was placed in between the light source and the cells. Additional cooling was provided by putting the cells on ice during the irradiation. The medium of cells irradiated outside the incubator was replaced every 2h and contained an additional buffer (20 mM MOPS, pH7).
**ROS production**

Cells were treated with 5 µM MitoSOX Red ROS probe (Molecular Probes) for 30 min at 37°C. After washing with PBS, the cells were collected and mitochondrial O₂•⁻ was detected by flow cytometry (BD LSR-II, BD Biosciences) using a 355 nm UV-laser in combination with a 575/26 nm filter (34). As a negative or positive control respectively, cells were treated with 100 µM menadione for 1h at 37°C either with or without an O/N pretreatment with 0.82 mg/ml N-acetyl cysteine (NAC).

**Western blotting**

BEAS-2B cells were left untreated or treated for 3h with 250 µM H₂O₂. After treatment, total protein extracts were collected in RIPA buffer (Thermo Scientific). Protein quantification was performed with the DC BioRad Protein Assay (BioRad). 30 µg protein was loaded on a 15% SDS-PAGE gel. Blots were blocked for 1h with 5% skimmed milk/TBS (for H3 core) or 5% bovine serum albumin (BSA)/TBS (for H3K4me3). For detection, the following antibodies were used in previously mentioned blocking solution with the addition of 0.1% Tween 20: 1:5000 rabbit anti-H3K4me3 (#07-473, Millipore), 1:4000 rabbit anti-H3 core (Ab1791, Abcam), and 1:3000 horseradish peroxidase-conjugated swine anti-rabbit (P0217, Dako). Western blot signal was generated with Pierce ECL Plus Western blot substrate (Thermo Scientific) and detected with the Biorad ChemiDoc MP imaging system (Biorad). Western blots were analyzed with the Image Lab 5.0 software (Biorad).

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