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A novel combination of Class I fumarase enzymes and a metabolite (alpha-ketoglutarate) signal the DNA damage response in *E. coli*.

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Abstract

Class-II fumarases (Fumarate Hydratase, FH) are dual targeted enzymes, occurring in the mitochondria and cytosol of all eukaryotes. They are essential components in the DNA damage response and more specifically in protecting cells from DNA double strand breaks. Similarly, the Gram-positive Bacterium *Bacillus subtilis* Class-II fumarase, in addition to its role in the TCA cycle, also participates in the DDR. *Escherichia coli*, harbors three fumarase genes; Class-I *fumA* and *fumB* and Class-II *fumC*. Notably, Class-I, show no sequence similarity to Class-II and are of different evolutionary origin. Strikingly, here we show that *E. coli* fumarase functions are distributed between Class-I fumarases which participate in the DDR, and the Class-II fumarase which participate in respiration. In *E. coli*, we discover that the signaling molecule, alpha-ketoglutarate (α-KG), has a novel function in *E. coli*, complementing DNA damage sensitivity of *fum* null mutants. Excitingly, we identify the *E. coli* DNA repair enzyme AlkB, is a target of this α-KG signaling. In addition to α-KG, fumarate is shown here to affect DNA damage repair on two different levels, first by directly inhibiting the DNA damage repair enzyme AlkB demethylase activity both in vitro and in vivo (countering α-KG). The second is a more global effect on transcription, as *fum* null mutants exhibit a decrease in transcription of key DNA damage repair genes. Together these results show evolutionary adaptable metabolic signaling of the DDR in which fumarases and different metabolites are recruited regardless of the evolutionary Class preforming the function.

Significance Statement

Class-II fumarases have been shown to participate in cellular respiration and the DNA damage response. Here we show, for the first time, that in the model prokaryote, *Escherichia coli*, which harbors both Class-I and Class-II fumarases, it is the Class-I fumarases that participate in DNA damage repair by a mechanism which is different than

those described for other fumarases. Strikingly, this mechanism employs a novel signaling molecule, alpha-ketoglutarate (α-KG), and its target is the DNA damage repair enzyme AlkB. In addition, we show that fumarase precursor metabolites, fumarate and succinate, can inhibit the α-KG-dependent DNA damage repair enzyme, AlkB, both in vitro and in vivo. This study provides a new perspective on the function and evolution of metabolic signaling.

Main Text

Introduction

Fumarase (fumarate hydratase, FH) is an enzyme that participates in the tricarboxylic acid (TCA) cycle, there, it catalyzes the reversible hydration of fumarate to L-malate [1]. In eukaryotes, in addition to its mitochondrial localization, a common theme conserved from yeast to humans is the existence of a cytosolic form of fumarase [2, 3]. The dual distribution of fumarase between the mitochondria and the cytosol is a universal trait, but intriguingly, the mechanism by which dual distribution occurs does not appear to be conserved [4-8]. We discovered that in eukaryotes the cytosolic form of this TCA enzyme was shown to have an unexpected function in the DNA damage response (DDR), and specifically, a role in recovery from DNA double strand breaks (DSBs) [9]. It appears that metabolic signaling in these organisms is achieved via the fumarase organic acid substrate fumarate [10, 11]. A recent study by Singer et al. [12] shows that the Gram-positive *Bacillus subtilis* bacterial fumarase, has a role in the TCA cycle, yet also participates in the DDR. Fumarase dependent signaling of the DDR is achieved by its metabolic product, L-malate, which affects RecN (the first protein recruited to DNA damage sites) at the level of expression levels and localization. That study indicated that

the dual function of this enzyme predated its dual distribution and, according to our model, was the driving force for the evolution of dual targeting in eukaryotes [12].

An intriguing variation to the themes above, is the fact that there are two distinct classes of fumarase. In the organisms mentioned above (*Saccharomyces cerevisiae*, human, *B. subtilis*) the fumarase that participates in both the TCA cycle and in DNA repair, belongs to the Class-II fumarases. In contrast, the Class-I fumarases, which bear no sequence or structural similarity to Class-II fumarases, is predominantly found in prokaryotes and in early evolutionary divergents in the eukaryotic kingdom (protozoa and invertebrates) [1].

The model organism that we have employed in this study is the Gram-negative bacterium *E. coli* which harbors three fumarase genes; Class-I, *fumA* and *fumB* and Class-II, *fumC*. FumA and FumB are homologous proteins, sharing 90% amino acid sequence identity. Both FumA and FumB kinetic parameters are very similar with respect to the natural substrates L-malate and fumarate [13]. FumC is homologous to eukaryotic and *B. subtilis* fumarases, sharing 64% amino acid sequence identity with eukaryotic and *B. subtilis* fumarases.

Employing the *E. coli* model, we ask whether Class-I and/or Class-II fumarases are involved in the DDR and/or the TCA cycle and what is the distribution of functions between these two Classes of fumarases in this organism. Here we show that FumA and FumB are participants of the DDR in *E. coli*, while FumC naturally participates in respiration. FumA and FumB participation in the DDR is based on a fascinating interplay of TCA cycle Intermediates, alpha-ketoglutarate (α-KG), fumarate and succinate. In *E. coli* the absence of fumarase is shown to affect the transcription of many genes following DNA damage induction, including many DNA repair genes. Specifically, we show that the target of alpha-ketoglutarate is the α-KG-dependent DNA repair enzyme AlkB

demethylase which is required for successful repair of DNA damage and which is also modulated by fumarate and succinate.

Results

ΔfumA and *ΔfumB* but not *ΔfumC E. coli* strains, are sensitive to DNA damage induction.

In order to examine a possible role for *E. coli* fumarases in the DNA damage response, we induced DNA damage in *E. coli* strains lacking each of the fumarase genes, by exposing them to treatment with Ionizing radiation (IR, Fig. 1A) or methyl methanesulfonate (Fig. 1B, MMS, 0.35% [v/v] for 30 or 45 minutes). Figures 1A and 1B (compare rows 2 and 3 in each panel to row 1) demonstrate that compared to the control (WT), strains lacking *fumA* or *fumB* are very sensitive to DNA damage (IR or 0.35% [v/v] MMS for 45 minutes), while the strain lacking *fumC* seems unaffected by the DNA damage induction (row 4). These results suggest that FumA and FumB play a role in the DNA damage response, while FumC has no observable role in this process. While the levels of FumA exhibit a very insignificant change in protein levels upon treatment with MMS (Fig 1C top panel), the levels of FumC increase (Fig 1C second panel), thereby revealing a more complex picture of regulation. Intriguingly, the double mutant (Fig 1D, middle panel, *ΔfumAB,* Row 5) is resistant to DNA damage induction, either IR or MMS treatment and shows a phenotype resembling the WT strain (Row 1), when compared to single *fumA* and *fumB* deletion strains (*ΔfumA, ΔfumB,* rows 2 and 3) or a triple mutant lacking all three fumarase genes (*ΔfumACB,* row 6) which are sensitive to IR and MMS. One possible explanation for this phenomenon is that in the absence of FumA and FumB, FumC expression in the *ΔfumAB* strain (i.e. no MMS treatment) increases to undertake both roles of respiration and participation in the DDR (Fig 1E).

Worth mentioning here and referred to in the next section, is that complementation of the DNA damage sensitivity can be achieved by expression of the genes in trans (Fig S1B, 3).

All three *E coli* fumarase genes can participate in the TCA cycle.

It is important to emphasize, with regard to respiration, that a functional TCA cycle including participation of fumarase is required (Figure 1D, right panel). Accordingly, we find growth of our strains (WT, *ΔfumA*, *ΔfumB, ΔfumC* and *ΔfumAB)* which harbor at least one functional fumarase gene, on minimal media with acetate as a sole carbon and energy source. In contrast the triple mutant (*ΔfumACB*), completely lacking fumarase, is unable to respire and therefore unable to grow on this media (Figure 1D, right panel row 6). The Δ*fumC* mutant shows an interesting respiration phenotype, although FumA and/or FumB seem to participate primarily in the DDR, it would seem that in the absence of FumC, these enzymes have the ability to participate in respiration, but likely play a less critical role in this function. Figure 1F shows the relative enzymatic activity of each of the mutant strains compared to the WT. These strains were assayed at 250nm for fumarate production using L-malate as a substrate, and show an observable decrease in enzymatic activity of fumarase mutant strains when compared to the WT (*ΔfumA* 50%, *ΔfumB* 30%, *ΔfumC* 90%, *ΔfumAB* 50% and *ΔfumACB* with insignificant activity). After treatment with MMS, the WT exhibits a 40% reduction in enzymatic activity and the mutants also exhibit an additional reduction in enzymatic activity (e.g. *ΔfumA 10%, ΔfumB 15%, ΔfumAB 5%)* when compared to the same untreated strain. The sharp decrease of fumarase activity in ***ΔfumC*** indicates that FumC is responsible for most of the fumarase activity in *E. coli*. FumA and FumC protein levels of these strains under the

same conditions are shown in figure S1A. These strains also show an expected decrease in FumC protein levels in the mutants compared to the WT strain, coupled with further reduction in FumC levels following MMS treatment (with the exception of the Δ*fumAB* strain as shown above). FumA levels in Δ*fumB* and Δ*fumC* strains resemble the FumA levels observed in the WT strain before induction of DNA damage, but show more significant reduction in FumA levels compared to the WT strain following MMS treatment.

As controls for the above experiments, we show that over-expression of FumA, FumB or FumC from high copy number plasmids (pUC-18) in the triple fumarase mutant (*ΔfumACB*) brings about a partial complementation of DNA damage sensitivity while only FumC appears to fully complement respiration (Fig. S1B). The expression levels of each of these constructs is shown in Fig. S1C. Due to the fact that these experiments are not under natural induction, but rather under over-expression conditions, FumC seems to have the same effect as FumA and FumB in regard to DNA damage induction. Nevertheless, it is also evident that under conditions of *fumA* and *fumB* absence, FumC, which is then overexpressed and takes over this role to participate in the DDR.

E. coli fumarases function in both the DDR and respiration in a yeast model system.

In order to validate that indeed FumA and FumB participate in the DNA damage response and FumC participates in respiration, we employed, as in the past [9, 12], the yeast *S. cerevisiae* as a model. We cloned each of these genes into yeast expression vectors and transformed them into two different yeast strains. The first yeast strain, Fum1M, harbors a chromosomal *fum1* deletion (*Δfum1*) and a *FUM1* open reading frame insertion in the mitochondrial DNA. This allows exclusive mitochondrial fumarase

expression, lacking extra-mitochondrial (cytosolic/nuclear) fumarase [9]. This Fum1M strain which is sensitive to DNA damage was transformed with each of the fumarases separately (Fig. 2A) in order to test whether bacterial FumA and FumB are able to protect against DNA damage, a function of the cytosolic yeast fumarase. To this end, we induced DNA damage by IR as shown in Fig. 2A or hydroxyurea (HU, not shown). Compared to the WT and Fum1M strains (rows 1 and 2 respectively), *E. coli* FumA and FumB are able to protect yeast from DNA damage while FumC does not (rows 3, 4 and 5 respectively).

The second yeast strain completely lacks fumarase *(Δfum1*) and therefore lacks respiration ability. We transformed each of the fumarase genes separately (Fig. 2B) and growth was followed on synthetic complete (SC) medium supplemented with ethanol (as a sole carbon and energy source). Compared to the Δ*fum* strain that lacks respiration ability (Fig. 2B, compare row 2 to row 1), and to the Δ*fum* that harbors yeast Fum1 encoded from a plasmid (row 3), FumA and FumB are unable to complement respiration while FumC partially complements respiration (rows 4, 5 and 6 respectively). The expression levels of each of these constructs is similar relative to the loading control aconitase (Fig. 2C). As FumA and FumB harbor no structural similarity to the yeast fumarase, this result does not definitively mean that they do not participate in respiration, but that due to evolutionary structural incompatibility with other cellular machineries, these enzymes are not able to complement respiration in this higher organism.

Previous unpublished work from our lab [14], demonstrated that fusion of the fumC coding sequence to *Neurospora crassa* F_o-ATPase, Su9 mitochondrial targeting sequence, led to complete import and processing of *E. coli* FumC in mitochondria. Without this sequence, FumC is not in mitochondria, but can complement yeast *fum* null mutants in cell respiration due to transport of the organic acid substrates (fumarate and

L-malate) in and out of the organelle. The complementation of DNA damage sensitivity by FumA and FumB indicates that these enzymes indeed participate in the DDR and this function is evolutionary conserved [12], while FumC participates in the TCA cycle and respiration in *S. cerevisiae*.

FumC is homologous to eukaryotic fumarase and to *B. subtilis* fumarase, and as referred to above, the cytosolic role of these fumarases is in the DDR. Intriguingly, in *E. coli* this role is carried out by FumA and FumB, although they have no sequence or structural similarity to the Class-II fumarases, besides their activity. To challenge this apparent distribution of functions between *E. coli* Class-I and Class-II fumarases and, in particular, the inability of FumC to function in the DDR, we asked whether this is due to a problem in the localization of FumC in the nucleus. As previous research shows that upon DNA damage induction there is migration of fumarase to the nucleus, there it participates in various DNA damage repair pathways [9, 10], the approach we took was to force the FumC protein into the yeast nucleus. We constructed a *fumC* ORF fused to a nuclear localization sequence (NLS). The modified protein was expressed in a yeast Fum1M strain and the respective strains were grown with or without HU (Fig 2D). We found that the modified FumC, containing an NLS was capable of complementing the Fum1M sensitivity to HU (Fig 2D, row 5). As controls we show that the original FumC, a FumC that harbors a nuclear export signal (NES) (row 4) or a FumC harboring a mutated NLS (mNLS, row 6) (Fig. 2D) do not complement Fum1M strain sensitivity to HU. The expression levels of each of these constructs is similar relative to the loading control aconitase (Fig. 2E). The NES sequence once fused to FumA (FumA-NES) or FumB (FumB-NES), render these enzymes completely incapable of protecting fum1M against DNA damage (Fig. S2A, S2B). Figure S2C depicts expression of these modified proteins. Thus, localization of fumarase to the nucleus is required for its DDR function.

FumA and FumB enzymatic activity is required for their DNA damage response function.

We have previously shown that the enzymatic activity of Class-II fumarases is required for their role in the DNA damage response [9, 12]. In order to ask whether the same is true regarding Class-I enzymes, FumA and FumB, we first needed to obtain enzymatically compromised mutants of these proteins. For this, we designed substitution mutations based on the predicted structure of *E. coli* Class-I fumarases according to the solved crystal structure of Class-I FH of *Leishmania major* (LmFH). We focused on the most conserved amino acids within a pocket that is suggested to be important for its catalytic activity [15]. Figure 3A depicts a dimeric homology model of the proposed structure for either FumA or FumB proteins which share 90% amino acid identity, and it is believed that they possess the same structure. The residues shown in spherical mode represent the dimeric enzyme interface that together form a cavity that in LmFH leads to the active site. Figure 3B shows alignment of the proposed *E. coli* Class-I fumarase structure (green) with that of *L. major* (blue, red, orange and yellow). We cloned PCR products harboring the point mutations into appropriate *E. coli* expression vectors. These plasmids were transformed into *E. coli* strains lacking each of the respective genes (Fig. 3C, 3D) and their expression was examined by Western blot (3E). Upon exposure to DNA damage (0.35% [v/v] MMS, 45 min), we observed an inability of the variant enzymes to protect against DNA damage in *E. coli* (Fig. 3C and 3D, compare rows 4 and 5 to row 3). The fumarase null mutant strain harboring these plasmids exhibits a highly significant reduction in enzymatic activity compared to wild-type FumA and FumB (Fig. 3F, 3G, compare bars 3 and 4 to bars 2). These results indicate that the enzymatic

activity of FumA and FumB is crucial for their DDR related functions of fumarase in *E. coli*.

Alpha-Ketoglutarate complements DNA damage sensitivity of *E. coli fum* null mutants.

In *S. cerevisiae* and human cells, the lack of extra mitochondrial fumarase and resulting sensitivity to DNA damage, can be complemented by externally added fumarate (fumaric acid, in the form of an ester, mono-ethyl fumarate, which is cleaved in the cells to form the free acid) [9, 10]. In *B. subtilis* the sensitivity of a fumarase null mutant to DNA damage, can be complemented by L-malate [12]. Both fumarate and L-malate are fumarase related enzymatic substrates/products. To examine if fumarase associated metabolites (substrates or products) complement the lack of fumarase in the DDR of *E. coli*, bacteria were grown in the presence of 25mM TCA cycle organic acids, added to the medium. An amazing result, as shown in Figure 4, is that *E. coli* strains deleted for the *fumA*, *fumB* and all three *fum* genes are protected from MMS induced DNA damage (0.35% [v/v] MMS, 45 min) by α-KG (bottom panel, compare rows 2, 3 and 6 to rows 1 and 4) and not by fumarate or L-malate (panels 5 and 6 respectively). This complementation can be achieved by concentrations as low as 1mM α-KG added to the growth medium (data not shown). Growth of these strains on LB metabolite plates without MMS treatment, shows growth consistent with that of the controls (top panel). This α-KG complementation of DNA damage sensitivity is exclusive to *fum* null mutants, as it does not protect *E. coli* strains harboring deletions in other DNA damage response genes (Fig S3) or composite mutants of DDR genes and *fumA*/*fumB* from DNA damage induction, suggesting a direct relationship between fumarase and α-KG within the DNA damage response of *E. coli*.

To investigate this relationship, we decided to study the α-KG effect on two different levels; the first being to investigate a global impact of α-KG on transcription and induction of DNA repair pathways in *E. coli*. The second was to investigate a direct impact on the activity of DNA repair related proteins.

Absence of *E. coli* fumarases affects the transcript levels of many genes including adaptive and SOS response genes <u>but does not involve α-KG signaling</u>.

RNA-seq analysis detected differentially expressed genes (DEGs) in the *fum* null mutant strains compared to the WT strain in response to MMS treatment. These results are described in detail in the supplementary material (supplementary figure S4). Analyzing DEGs altered exclusively in the WT strain or exclusively in either *fum* mutant strains, yielded no candidate genes that may explain the DNA damage sensitivity of *ΔfumA, ΔfumB* and *ΔfumACB*, as all DNA repair systems are induced in all MMS treated strains, though, worth mentioning is that the absence of fumarase lead to lower expression of key DNA damage repair enzymes. It is important to emphasize that <u>α-KG has no additive effect to treatment with MMS alone</u> on the expression levels of these genes, and that fumarase absence and or the fumarate concentration effect on the expression level of these key DNA damage repair enzymes, is still not fully known.

Alpha-ketoglutarate enhances, whereas succinate and fumarate inhibit AlkB DNA damage repair activity.

Our most important conclusion from the previous section is that the candidate DNA damage signaling metabolite, α-KG, does not function at the level of transcription. Given these results, we chose to focus on DNA repair enzymes whose activity may be regulated by TCA cycle organic acids. We decided to focus on AlkB due to its known

function in DNA damage repair and its interaction with α-KG. AlkB is a Fe-(II)/α-KG-dependent dioxygenase in *E. coli* which catalyzes the direct reversal of alkylation damage to DNA, primarily 1-methyladenine (1mA) and 3-methylcytosine (3mC) lesions. A hallmark of AlkB-mediated oxidative demethylation is that the oxidized methyl group is removed as formaldehyde [16]. Previously published research in the bacterium *B. subtilis* showed that in fumarase null mutants, there is an accumulation of succinate and fumarate [12]. In eukaryotes, fumarate and succinate have been shown to inhibit human α-KG-dependent histone and DNA demethylases and in yeast, fumarate inhibits the sensitivity of components of the homologous recombination repair system to DNA damage induction [10, 11, 17]. The inhibition of histone and DNA α-KG-dependent demethylases by fumarate and succinate was established as competitive, based on their structural similarity to α-KG [17].

To test whether fumarate and succinate inhibit AlkB demethylase activity in *E. coli*, we employed two previously published protocols [18, 19] for in vitro measurement of DNA damage repair by AlkB in the presence and absence of succinate, fumarate and malate. Such an effect, if found could explain the increase in sensitivity of *fum* null mutants to MMS generated DNA damage.

The first approach was direct fluorescence-based formaldehyde detection using acetoacetanilide and ammonium acetate, which together form an enamine-type intermediate. This intermediate undergoes cyclodehydration to generate highly fluorescent dihydropyridine derivative, having maximum excitation at 365 nm and maximum emission at 465 nm [19]. A 70bp oligonucleotide (see materials and methods) was treated with MMS to allow methylation, and was directly incubated with purified AlkB to facilitate DNA repair. DNA repair was measured by release of formaldehyde (RFU

[HCHO concentration]). While α-KG is required for the full activity of AlkB in vitro (Figure 5A, bar 1, 200μM of α-KG), succinate and fumarate competitively inhibit E. coli AlkB demethylase activity in a dose dependent manner (compare bars 2-4, 5-7 to bar 1) (200μM of succinate and fumarate with increasing amounts of α-KG). Malate appears to have an insignificant, non-dose dependent, effect on AlkB demethylase activity (bars 8-10) (200μM of malate with increasing amounts of α-KG).

The second approach, employs a restriction enzyme-based demethylation assay [18]. The same substrate (70bp oligonucleotide substrate that contains the MboI restriction enzyme recognition site in the middle of the sequence) was treated with MMS to allow methylation, and was directly incubated with purified AlkB to facilitate DNA repair. By digestion with the dam methylation sensitive enzyme MboI, successful repair of methylated oligonucleotide (complete demethylation by AlkB) yields a 35bp product (Fig 5B, compare lane 4 to lane 3 [methylated dsDNA [me-dsDNA] undigested by MboI] and lane 2 [control un-methylated dsDNA digested by MboI]). Shown in Figure 6B, is the inhibition of AlkB demethylase activity which can be detected by the non-apparent 35bp fragment. Succinate and fumarate inhibit AlkB in a dose dependent manner (compare lanes 5-8 [containing 200 μM succinic acid and increasing concentrations of α-KG] and lanes 9-12 [200 μM fumaric acid and increasing concentrations of α-KG] to lane 4 [containing 200 μM α-KG]). Malate appears to have an insignificant effect on AlkB demethylase activity (lanes 13-16). Together, these results demonstrate, for the first time, *E. coli* AlkB inhibition by fumarate and succinate.

In order to validate these results in vivo, an AlkB inhibition experiment with high molar concentrations (25mM) of fumarate and succinate was carried out. For this purpose, we induced DNA damage (using 0.35% [v/v] MMS, 45 min) in an *E. coli* strain lacking *alkB* (*ΔalkB*), and in a *ΔalkB* strain, overexpressing AlkB from a plasmid. Figure 5C shows

that *ΔalkB* is extremely sensitive to MMS generated DNA damage, and overexpression of AlkB completely compensates for this DNA damage sensitivity phenotype (panel 2, compare row 3 to row 2). Fumarate and succinate exhibit a strong inhibition of AlkB ability to repair damaged DNA (compare top row, panels 4 and 5 to panels 2 and 3). Addition of as low as 1 mM of α-KG complements AlkB activity in vivo (compare bottom row, panels 2 and 3 to top row panels 4 and 5). These results are consistent with the previously shown in vitro experiments above, in which fumarate and succinate strongly inhibit AlkB DNA repair activity with malate having no effect on the AlkB DNA repair function. These results are summarized in a model in which the inhibition of α-KG-dependent-AlkB by accumulation in vivo of fumarate and succinate leads to the sensitivity of *ΔfumA, ΔfumB* and *ΔfumACB* to DNA damage (Fig. 5D).

Discussion

Recent discoveries identify the recruitment of metabolic intermediates and their associated pathways in the signaling of crucial cellular functions. The best example is the enzyme fumarase (fumarate hydratase) which belongs to Class-II (FumC-like fumarases). This TCA cycle enzyme has been shown to be involved in the DNA damage response in yeast and human, and more recently in the Gram-positive bacterium *B. subtilis* [10-12, 20]. In human and yeast, fumarate (fumaric acid) is the signaling molecule targeting histone demethylases and a HRR resection enzyme respectively [10, 11]. In *B. subtilis* L-malate is the signaling molecule, modulating RecN (the first enzyme recruited to DNA damage sites) expression levels and cellular localization [12].

In this study we show an amazing variation on the above themes by studying *E. coli*, which harbors the two Classes of fumarases, Class-I *fumA* and *fumB* and Class-II *fumC*. At first glance, contrary to previous work on Class-II fumarases, *E. coli* FumC does not

have a natural role in the DDR, while FumA and FumB, harboring no structural or sequence similarity to the Class-II fumarases, are participants in the DDR in this Gram-negative bacterium. In this regard, not only do FumA and FumB participate in the DDR in *E. coli*, they can substitute for yeast fumarase in a Fum1M DNA damage sensitive model strain [9, 12]. This shows that Class-I fumarases, FumA/FumB, that are non-homologous to the yeast Class-II fumarase, can function in the DDR, indicating that not the enzyme is needed for this DDR related function, but rather its enzymatic activity and related metabolites. As we have demonstrated for Class-II FH in human cell lines, yeast and *B. subtilis*, Class-I FumA and FumB enzymatic activity is crucial for their DNA damage protective function. This is supported in this study by the fact that enzymatically compromised variants of the Class-I fumarase do not signal the DDR. Our results indicate that it is only the level of fumarase activity that is important, since if over-expressed all three *fum* genes can function in the DDR in *E. coli*.

Most interesting is, that neither fumarate nor malate added to the growth medium are capable of complementing the DNA damage sensitive phenotype of *fum* null mutants as demonstrated for the previously studied organisms. α-KG, a TCA cycle intermediate with no direct interaction with fumarase appears to be the signaling metabolite. Moreover, this α-KG mediated protective effect seems to be exclusive to *fum* null mutants, as the sensitivity phenotype of other *E. coli* null mutants in DNA damage repair genes, is not alleviated by the addition of α-KG to the growth medium. Fumarase absence, and perhaps alteration of local concentration of TCA cycle metabolites specifically affects the activity of the DNA repair enzyme AlkB On the one hand, and leads to a reduced transcriptional induction of DNA damage repair components, on the other.

The absence of fumarase is shown here to affect AlkB, a Fe-(II)/α-KG-dependent dioxygenase via its precursor metabolites succinate and fumarate. Fe-(II)/α-KG-

dependent dioxygenases are present in all living organisms and catalyze hydroxylation reactions on a diverse set of substrates, including proteins, alkylated DNA/RNA, lipids and others [21, 22]. Fumarate and its precursor succinate have been shown to inhibit a variety of Fe-(II)/α-KG-dependent dioxygenases, and this inhibition was shown to antagonize α-KG-dependent processes and negatively regulate Fe-(II)/α-KG-dependent dioxygenases such as prolyl hydroxylases (PHDs), histone lysine demethylases (KDMs), collagen prolyl-4-hydroxylases, and the TET (ten-eleven translocation) family of 5-methlycytosine (5mC) hydroxylases [10, 17, 20, 23-26]. We find, in agreement with the above observations, that AlkB enzymatic activity is inhibited by the presence of high concentrations of fumarate and succinate both in vitro and in vivo, indicating that AlkB inhibition has a specific consequence in vivo, leading to the sensitivity of *ΔfumA, ΔfumB* and *ΔfumACB* to DNA damage.

The above findings suggest that the recruitment of metabolic intermediates and their associated pathways in signaling during evolution, was adaptable. In an ongoing study we are examining the distribution of Class-I and Class-II fumarases through the different domains of life. Class-I and Class-II can be found in prokaryotes and eukaryotes as sole fumarase representatives; e.g. mammals harbor only Class-II while many protozoa contain only Class-I fumarases. Some organisms, such as *E. coli*, harbor both Classes and, in fact, very few can be found that lack both Classes of fumarases. At this stage it is difficult for us to hypothesize why a certain metabolite, Class of enzyme, or pathway was chosen to signal the DDR, but this study gives us a new perception on how metabolites, metabolic enzymes and pathways could have been recruited during evolution of metabolite signaling.

There are many unanswered questions regarding the fumarases in *E. coli*, for example does subcellular localization of the fumarases or their metabolites play a role in their

DDR function? Using specific Class-I and Class-II antibodies we detect that the enzymes do not co-localize with the nucleoid but rather localize to the cell poles regardless of treatment with MMS (not shown). What governs the levels of α-KG and other metabolites at sites of DNA damage? And how exactly does fumarate exert its effect on transcription? Further studies are needed to address these complex questions and systems.

Materials and Methods

1. Bacterial Strains and Growth Conditions

Bacterial strains were provided by the Coli Genetic Stock Center and are listed in supplementary table 1. Additional strains were constructed using the lambda red system [27], plasmids were constructed using standard methods, plasmids and primers are listed in supplementary table 2. Bacteria were grown in Luria-Bertani (LB) broth to early to mid-exponential phase (OD_{600nm}=0.35), supplemented, when needed, with ampicillin (Amp, 100 g/ml) or 25 mM metabolites (α-KG, succinate, fumarate, malate [Sigma Aldrich]). MMS was added to a final concentration of 0.35% [v/v]. When indicated 0.5 mM of IPTG (isopropyl-D-thiogalactopyranoside, Sigma) was added. For agar plates, 2% agar was added.

2. Yeast Strains and Growth Conditions

S. cerevisiae strains used in this research are listed in supplementary table 3. *S. cerevisiae* strains used were grown on synthetic complete (SC) medium containing 0.67% (wt/vol) yeast nitrogen base +2% glucose (SC-Dex) or 2% galactose (SC-Gal) (wt/vol), supplemented with appropriate amino acids. For agar plates, 2% agar was added. HU was added to SC medium (supplemented with glucose) to a final concentration of 200 mM. The cells were grown overnight at 30°C in SC-Dex, washed in

sterile double distilled water and transferred to SC-Gal for induction of genes under GAL promoter.

3. Construction of mutants- Gene knockout using λ red system (pKD46)

3.1 Gene Disruption

Δ*fumAB*, Δ*fumACB* strains (and other composite mutants in this study) were constructed by the following: Gene disruption as previously described [27]. Essentially, Δ*fumB* and BW25113 Transformants carrying λ red plasmid were grown in 5-ml LB cultures with ampicillin and L-arabinose at 30°C to an OD_{600} of 0.6. The cells were made electrocompetent by concentrating 100-fold and washing three times with ice-cold sterile DDW. Electroporation was done by using a Cell-Porator with a voltage booster and 0.2-cm chambers according to the manufacturer's instructions (BIO-RAD). By using 100 µl of suspended cells and 100 ng of PCR product (for Δ*fumAB* strain cat cassette with flanking upstream and downstream sequences of *fumA*, and for Δ*fumAC* strain cat cassette (Chloramphenicol, Cm) with flanking upstream of *fumA* and downstream of *fumC*), the cells were added to 1-ml LB, incubated 3 hours at 37°C, and then spread onto agar supplemented with Cm/Kan to select CmR/KanR transformants. After primary selection, mutants were colony purified twice selectively at 37°C and then tested for loss of the parental gene by PCR.

Following verification of *fumAC* knock out and cassette insertion, a second Knock out was carried out with Kan (Kanamycin) cassette with flanking upstream and downstream of *fumB* [two step Gene knockout]).

3.2. PCR verification of gene disruption

Two PCR reactions were used to show that the mutants have the correct structure. A freshly isolated colony was used in separate PCRs following a 5-min pre-incubation ''hot start,'' at 95°C. One reaction was done by using nearby locus-specific primers (150bp

upstream of *fumA* or *fumB*) with the respective common test primer (c1 and k2) to verify gain of the new mutant specific fragment (antibiotic resistance), and a second reaction was carried out with the flanking locus-specific primers to verify simultaneous loss of the parental (non-mutant) fragment. Control colonies were always tested side-by-side.

4. Prediction of FumA and FumB structure

Prediction of FumA and FumB were as previously described [28]. Essentially, Homology models of the structure of *E. coli* FumA and FumB (based on the solved structures of 5L2R of *L. major*, [15]) were generated using I-TASSER [29]. The models with the highest probability score among the provided models were selected for further investigation. Using PeptiMap [30] for the identification of peptide binding sites and CONSURF [31] for conservation analysis of FumA and FumB amino acid sequence. Mutations were designed based on conserved amino acids within the peptide binding pockets detected. Figures of structures were generated using the PyMOL software (Schrödinger Inc).

4.1. Mutagenesis of suspected catalytic site

Three proposed amino acids suspected to be vital for the suspected catalytic site of FumA and FumB*;* Isoleucine 233, Threonine 236 and Tyrosine 481 were mutagenized by site directed mutagenesis with a KAPA HiFi HotStart PCR kit (KAPABIOSYSTEMS). Exchanging Tyrosine, at 481 with Aspartic acid (Y481D). We chose to combine I233 and T236 generating a double point mutation, exchanging Isoleucine at 233 for lysine, and exchanging Threonine at 236 with Isoleucine (I233K T236I). All mutated genes were cloned into Puc18 cloning vector and transformed into bacterial strains Δ*fumA*, Δ*fumB* and yeast strains Fum1M and Δ*fum1*.

5. FumA/FumB activity assay

Fumarase activity of FumA and FumB were assayed as previously described [13]. Essentially, fumarase enzymatic activity was measured by monitoring malate depletion at 250 nm spectrophotometrically in quartz cuvettes (Fumarase activity at 250 nm with L-malic acid as the substrate). Assays were performed in assay buffer (100 mM Hepes buffer pH 8 with 5% glycerol and 0.5 mM DTT).

8. AlkB in vitro demethylation assay

AlkB demethylation activity was measured by two previously described methods [18, 19]. For both methods 40 µg of chemically synthesized oligonucleotides (IDT) that contains the restriction site for the methylation sensitive restriction enzyme MboI (underlined) was used 5'-GGATGCCTTC GACACCTAGC TTTGTTAGGT CTGGATCCTC GAAATACAAA GATTGTACTG AGAGTGCACC-3'). The oligonucleotides were treated with MMS buffer (5% (v/v) MMS, 50% EtoH in a final volume of 500 µl in presence of 200 mM K_2HPO_4 at room temperature for 16 hours), dialyzed in TE buffer (10 mM Tris pH 8.0, 1 mM EDTA pH 8.0 [Sigma Aldrich E5134-50G]) using Spectra/Por dialysis membrane (MWCO: 3500) and precipitated with 2 volumes of ice cold 100% EtoH and 0.3M sodium acetate pH 5.5, washed twice with 70% EtoH and dissolved in DNase/RNase free water.

8.1 Immunoprecipitation of FLAG tagged AlkB-

AlkB was expressed in *E. coli* BL21 and Immunoprecipitated using FLAGIPT1 kit (sigma Aldrich) according to manufacturer's instructions, and Flag-AlkB proteins were eluted using a Flag peptide.

8.2 Direct fluorescence-based formaldehyde detection

This assay was performed as previously described [19]. Repair reactions (50 µl) were carried out at 37◦C for 1 hour in the presence of 1 µM AlkB, 0.5 µg (1 µM) methylated oligonucleotide and reaction buffer (20 mM Tris–HCl pH 8.0, 200 µM indicated metabolite [αKG acid, succinic acid, fumaric acid and malic acid], 2 mM L-Ascorbate and

20 µM $Fe(NH_4)_2(SO_4)_2$). Formaldehyde release was detected by mixing demethylation repair reaction product with 40 µl of 5 M ammonium acetate and 10 µl of 0.5 M acetoacetanilide to make the final volume 100 µl. The fluorescent compound was allowed to develop at room temperature for 15 min and then entire reaction mixture was transferred to 96-well microplate and analysed using a multimode reader setting the excitation wavelength at 365 nm and emission wavelength at 465 nm. Formaldehyde standard curve was prepared by selecting a range of pure formaldehyde concentrations from 2 to 20 µM.

8.3 Restriction based demethylation assay

This assay was performed as previously described [18]. Repair reactions (50 µl) were carried out at 37°C for 4 hours in the presence of 1 µM AlkB, 0.5 µg (1 µM) methylated oligonucleotide and reaction buffer (20 mM Tris–HCl pH 8.0, 200 µM indicated metabolite, 2 mM L-Ascorbate and 20 µM $Fe(NH_4)_2(SO_4)_2$). Repair reaction oligonucleotide was annealed to equi-molar complimentary ssDNA to generate methylated dsDNA in annealing buffer (50 mM Hepes pH 8 and 10 mM EDTA pH 8) at 37°C for 60 min. repaired dsDNA was digested by MboI restriction enzyme (2 hours at 37°C followed by heat inactivation). Digestion products were dissolved on 3% agarose containing SafeU (SYBR™ Safe DNA Gel Stain, Thermo Fisher Scientific, #S33102) with 10 mM sodium borate as electrophoresis buffer at 300 V for 20 min, and visualized using Gel Documentation System (Bio Rad).

9. Western blot analysis

E. coli cells were harvested in lysis buffer containing: 50 mM Tris pH 8, 10% glycerol, 0.1% triton, 100 mM PMSF (Sigma Aldrich 10837091001), 0.1 mg/ml Lysozyme (Sigma Aldrich 10837059001). Protein concentrations were determined using the Bradford method [32]. Loading amount on gels was normalized using protein concentration and

verified by recording the gels using Ponceau S staining. Samples were separated on 10% SDS-PAGE gels, transferred onto PVDF membranes (Millipore). The following primary antibodies were used: polyclonal anti FumA, polyclonal anti FumC and for identification of Class-I FH polyclonal anti FumB that recognizes both FumA and FumB (prepared in our lab).

S. Cerevisiae cells were harvested in lysis buffer containing: 10 mM Tris pH 8, 1 mM EDTA and 100 mM PMSF. Protein concentrations were determined using the Bradford method [33]. Samples were separated on 10% SDS-PAGE gels, transferred onto PVDF membranes (Millipore). The following primary antibodies were used: monoclonal anti FLAG (Sigma- F3165) and polyclonal anti Aconitase (prepared in our lab). All blots were incubated with the appropriate IgG-HRP-conjugated secondary antibody. Protein bands were visualized and developed using ECL immunoblotting detection system (ImageQuant LAS 4000 mini GE Healthcare) and Gel Documentation System (Bio Rad).

10. Statistical analysis

Statistical analysis was conducted with the two-tailed unpaired Student's t-test. All data represent the mean ± standard error of three independent experiments.

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Figures and Tables