AOP ID and Title:

SNAPSHOT

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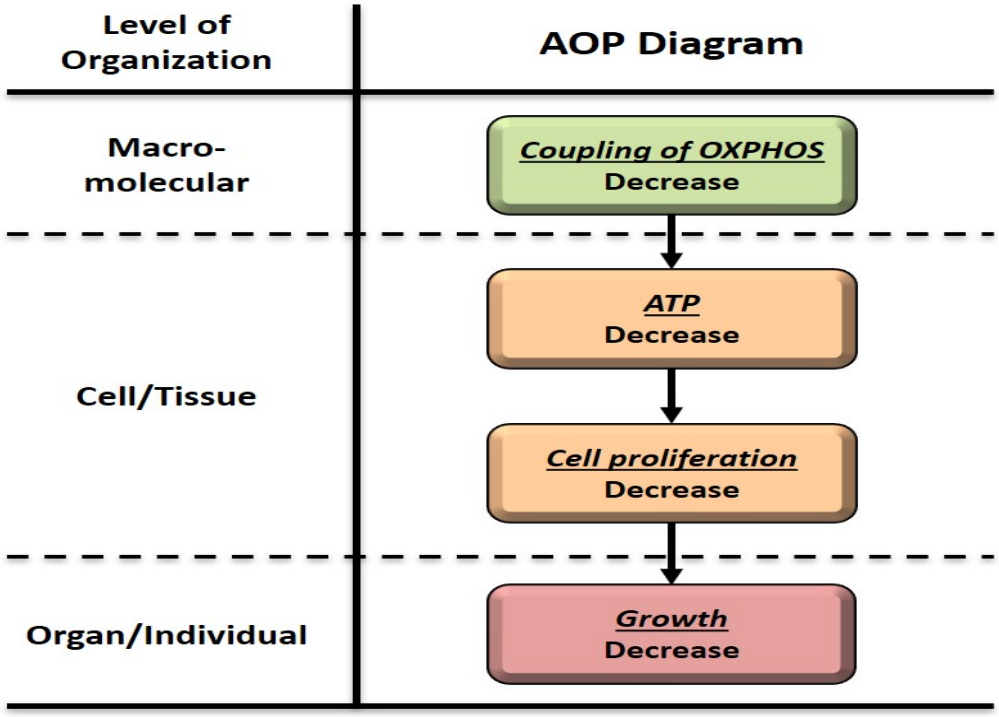
AOP 263: Uncoupling of oxidative phosphorylation leading to growth inhibition (1)

Short Title: Uncoupling of OXPHOS leading to growth inhibition (1)

Graphical Representation



Authors

You Song[a] and **Daniel L. Villeneuve**[b]

[a] Norwegian Institute for Water Research (NIVA), Gaustadalléen 21, NO-0349 Oslo, Norway

[b] U.S. Environmental Protection Agency, Great Lakes Toxicology and Ecology Division, Duluth, Minnesota 55804, USA

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Status

Author status	OECD status	OECD project	SAAOP status
Under development: Not open for comment. Do not cite			Under Development

Abstract

Uncoupling of oxidative phosphorylation (OXPHOS) is a well-known mechanism of action of many chemicals. Mitochondrial uncoupler-mediated energetic dysfunction is considered to affect growth, a common physiological process in most organisms and a highly regulatory relevant chronic toxicity endpoint widely included in the OECD test guidelines. This adverse outcome pathway (AOP) causally links uncoupling of OXPHOS to

growth inhibition, through ATP depletion and reduced cell proliferation as the intermediate key events (KEs), with strong weight of evidence support. The AOP is highly generalized with the intention of being applicable to a broad range of species groups, ranging from microalga to human. Three out of four KEs can be quantified using high-throughput methods, making this AOP particularly useful for screening, prioritization and hazard assessment of potential mitochondrial uncouplers and growth inhibiting chemicals. The AOP is also a core part of a larger network addressing uncoupling of OXPHOS mediated growth inhibition (AOP 263-268).

Background

The mitochondrial OXPHOS machinery is a key physiological process responsible for producing the primary cellular energy, adenosine triphosphate (ATP). During OXPHOS, a series of redox reactions (oxidation) are mediated by protein complexes in an electron transport chain to create a protonmotive force across the inner mitochondrial membrane (Liberman 1969). The PMF acts as a driving force of ATP synthesis through phosphorylation of adenosine diphosphate (ADP). The mitochondrial oxidation and phosphorylation are coupled to ensure continuous ATP supply for various physiological processes. A number of chemicals can bind to the inner mitochondrial membrane and dissipate the PMF, thus leading to uncoupling of OXPHOS and reduction in ATP synthetic efficiency. Classical “couplers” are normally protonophores with major characteristics of bulky hydrophobic moiety, an acid dissociable group and a strong electron-withdrawing group (Terada 1990). With the rapid development of *in silico* (Escher 2002; Attene-Ramos 2013; Attene-Ramos 2015; Xia 2018) approaches, more and more uncouplers have been identified. Their hazards and risks to the biota, however, remain to be assessed. Uncoupling of OXPHOS can affect many ATP-dependent biological functions. As a major process to achieve organismal growth, cell proliferation is positively correlated with the cellular ATP level and highly susceptible to energy depletion (Ramaiah 1964; Bonora 2012). Therefore, a link between uncoupling of OXPHOS and growth inhibition can be established with ATP depletion and reduced cell proliferation as the intermediate steps.

Summary of the AOP

Events

Molecular Initiating Events (MIE), Key Events (KE), Adverse Outcomes (AO)

Sequence	Type	Event ID	Title	Short name
	MIE	1446	Decrease, Coupling of oxidative phosphorylation (https://aopwiki.org/events/1446)	Decrease, Coupling of OXPHOS
	KE	1771	Decrease, Adenosine triphosphate (https://aopwiki.org/events/1771)	Decrease, ATP
	KE	1821	Decrease, Cell proliferation (https://aopwiki.org/events/1821)	Decrease, Cell proliferation
	AO	1521	Decrease, Growth (https://aopwiki.org/events/1521)	Decrease, Growth

Key Event Relationships

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Decrease, Coupling of oxidative phosphorylation (https://aopwiki.org/relationships/2203)	adjacent	Decrease, Adenosine triphosphate	High	High
Decrease, Adenosine triphosphate (https://aopwiki.org/relationships/2204)	adjacent	Decrease, Cell proliferation	Moderate	Moderate
Decrease, Cell proliferation (https://aopwiki.org/relationships/2205)	adjacent	Decrease, Growth	Moderate	Moderate

Stressors

Name	Evidence
2,4-Dinitrophenol	High
Pentachlorophenol	Moderate
Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone	High
Carbonyl cyanide m-chlorophenyl hydrazone	High
Triclosan	High
Dinoseb	Moderate
3,5-Dichlorophenol	Moderate
Emodin	High

Overall Assessment of the AOP

The weight of evidence assessment of the AOP was conducted based on the evolved Bradford-Hill considerations (Becker 2015) and according to the criteria in OECD's Guidance Document for Developing and Assessing AOPs (OECD 2018). Overall, the MIE and KE1 are scored as high due to good evidence to support their essentiality in the AOP, whereas KE2 is scored as moderate due to a lack of solid evidence to support its essentiality. The overall WoE of KER1 is considered high, as strong biological plausibility, empirical evidence and fairly good quantitative understanding were evidenced from multiple studies. The overall WoE of KER2 is considered moderate, due to high biological plausibility, acceptable empirical concordance and some biological understanding. The overall WoE of KER3 is scored as moderate, mainly due to good biological plausibility, whereas a lack of sufficient empirical evidence and quantitative understanding to further support the causality. The AOP is considered applicable to a wide range of species as well as a broad domain of chemicals. The rationales for making these judgements will be discussed in detail in the following sections.

Domain of Applicability

Life Stage Applicability

Life Stage	Evidence
Embryo	High

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
zebrafish	Danio rerio	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=7955)
Lemna minor	Lemna minor	Moderate	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=4472)
human	Homo sapiens	Moderate	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
mouse	Mus musculus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)
rat	Rattus norvegicus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)

Sex Applicability

Sex	Evidence
Unspecific	High

The **taxonomic application domain** of the AOP potential covers all animals, plants and some microorganisms such as fungus and protists, as mitochondrial OXPHOS is highly conserved in eukaryotes (Roger 2017).

The **life stage applicability domain** mainly contains embryos and juveniles, as growth is more relevant to developing organisms. It should be

noted that fully grown adults are also susceptible to uncouplers, as tissue/organ (e.g., adipose tissue) growth and regeneration still occur in adults (Yun 2015) and (Demine 2019). Classical uncoupler such as 2,4-DNP have been reported to cause weight loss in adult humans (Grundlingh 2011), suggesting that adults are partially in the applicability domain of this AOP.

The **sex applicability domain** is unspecific, as the AOP is mainly targeting growth effects in sextual immature organisms and the KEs are therefore harmonized between male and females. However, male and females may have different sensitivities to OXPHOS uncoupling, as the strategies for allocating energy for developmental processes may be gender specific (Demarest 2015). Follow-up studies may consider the inclusion of comparative analysis on gender susceptibility.

Chemical applicability domain: Weak acids, such as phenols, benzimidazoles, N-phenylanthranilates, salicylanilides, phenylhydrazones, salicylic acids, acyldithiocarbazates, cumarines, and aromatic amines are well-known protonophoric uncouplers. Classical uncouplers, such as 2,4-dinitrophenol (2,4-DNP), carbonyl cyanide-p-trifluoromethoxyphenyl hydrazone (FCCP), carbonyl cyanide m-chlorophenyl hydrazone (CCCP), pentachlorophenol (PCP), 3,5-dichlorophenol (3,5-DCP), 6-sec-butyl-2,4-dinitrophenol (dinoseb), SF 6847 (3,5-di-t-butyl-4-hydroxybenzylidinemalononitrile) have been widely used as positive controls in (eco)toxicological tests, whereas the hazards of “new” uncouplers, such as triclosan, emodin and metabolites of polybrominated diphenyl ethers (PBDEs) are also under extensive assessments. A number of potential uncouplers have been identified by *in silico* (Escher 2002; Attene-Ramos 2013; Attene-Ramos 2015; Xia 2018) approaches, and are considered in the chemical applicability domain of the AOP.

Essentiality of the Key Events

<table>
<tr><td rowspan="4">Support for Essentiality of KEs</td><td>Defining Question</td><td>What is the impact on downstream KEs and/or the AO if an upstream KE is modified or prevented?</td></tr>
<tr><td>High</td><td>Direct evidence from specifically designed experimental studies illustrating prevention or impact on downstream KEs and/or the AO if upstream KEs are blocked or modified.</td></tr>
<tr><td>Moderate</td><td>Indirect evidence that modification of one or more upstream KEs is associated with a corresponding (increase or decrease) in the magnitude or frequency of downstream KEs.</td></tr>
<tr><td>Low</td><td>No or contradictory experimental evidence of the essentiality of any of the KEs.</td></tr>
<tr><td>MIE:
(Decrease, Coupling of OXPHOS)</td><td colspan="2">Essentiality of the MIE is high.
Rationale:
1. Removal of the classical uncoupler carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) led to recovery of both MMP and ATP in rat cerebellar granule cells (Weisová 2012).
2. In the red abalone (Haliotis rufescens) larvae, removal of the uncoupler pentachlorophenol also led to recovery of the ATP level (Shofer 2002).
3. Addition of the recoupler GDP led to a rapid increase in ATP/ADP ratio in isolated guinea pig brown-adipose-tissue mitochondria where high activities of natural coupling by the UCPs were expected (Rafael 1976).
4. Addition of octanoate to 2,4-DNP exposed rat hepatocytes mitigated the uncoupling effect and partial restored the ATP/ADP ratio (Sibille 1995).</td></tr>
<tr><td>KE1:
(Decrease, ATP)</td><td colspan="2">Essentiality of KE1 is high.
Rationale:
1. One inhibition-rescue type of study clearly showed that addition of ATP re-stimulated proliferation in human lung adenocarcinoma cells pretreated with the uncoupler emodin (Wang 2017), hence providing direct evidence to support the essentiality of this KE.
2. Positive correlations between uncoupler mediated ATP depletion and reduced cell proliferation have also been documented by multiple studies (Sweet 1999; Fine 2009; Guimarães 2012; Sugiyama 2019).</td></tr>
<tr><td>KE2:
(Decrease, Cell proliferation)</td><td colspan="2">Essentiality of KE2 is moderate.
Rationale: there is a lack of direct evidence from specifically designed exposure-recovery or inhibition-rescue studies. However, it is well-known that tissue or organismal growth is achieved through cell proliferation.
1. Indirect evidence can also be obtained from a limited number of relevant studies showing a positive role of cell proliferation in mammalian tumor (Figarola 2018) or zebrafish embryo growth (Bestman 2015).</td></tr>
</table>

Weight of Evidence Summary

Biological plausibility

Support for Biological Plausibility of KERs	**Defining Question**	**Is there a mechanistic (i.e., structural or functional) relationship between KE_{up} and KE_{down} consistent with established biological knowledge?**
	High	**Extensive understanding based on extensive previous documentation and broad acceptance -Established mechanistic basis.**
	Moderate	**The KER is plausible based on analogy to accepted biological relationships but scientific understanding is not completely established.**
	Low	**There is empirical support for a statistical association between KEs, but the structural or functional relationship between them is not understood.**
MIE => KE1: **(Decrease, Coupling of OXPHOS leads to Decrease, ATP)**	**Biological Plausibility of MIE => KE1 is** high. **Rationale:** In eukaryotic cells, the major metabolic pathways responsible for ATP production are OXPHOS, citric acid (TCA) cycle, glycolysis and photosynthesis. Oxidative phosphorylation is much (theoretically 15-18 times) more efficient than the rest due to high energy derived from oxygen during aerobic respiration (Schmidt-Rohr 2020). As the ATP level is relatively balanced between production and consumption (Bonora 2012), ATP depletion is a plausible consequence of reduced ATP synthetic efficiency following uncoupling of OXPHOS.	
KE1 => KE2: **(Decrease, ATP leads to Decrease, Cell proliferation)**	**Biological Plausibility of KE1 => KE2 is** high. **Rationale:** Cell proliferation is a well-known ATP-dependent process. Cell division processes, such as the mitotic cell cycle uses ATP for chromosome movements and DNA replication (Kingston 1999). The synthetic processes of major cellular components that are necessary for cell structure and growth, such as proteins and lipids, also require sufficient ATP supply (Bonora 2012). Depletion of ATP therefore has a negative impact on these processes.	
KE2 => KE3: **(Decrease, Cell proliferation leads to Decrease, Growth)**	**Biological Plausibility of KE2 => KE3 is** high. **Rationale:** The biological causality between cell proliferation and growth has also been well established. It is commonly accepted that the size of an organism, organ or tissue is dependent on the total number and volume of the cells it contains, and the amount of extracellular matrix and fluids (Conlon 1999). Impairment to cell proliferation can logically affect tissue and organismal growth.	

Empirical support

Empirical Support for KERs	**Defining Question**	**Does KE_{up} occur at lower doses and earlier time points than KE down and at the same dose of stressor, is the incidence of KE_{up} >than that for KE_{down}? Are there inconsistencies in empirical support across taxa, species and stressors that don't align with expected pattern for hypothesized AOP?**
	High	**Multiple studies showing dependent change in both events following exposure to a wide range of specific stressors. (Extensive evidence for temporal, dose- response and incidence concordance) and no or few critical data gaps or conflicting data.**
	Moderate	**Demonstrated dependent change in both events following exposure to a small number of specific stressors and some evidence inconsistent with expected pattern that can be explained by factors such as experimental design, technical considerations, differences among laboratories, etc.**
	Low	**Limited or no studies reporting dependent change in both events following exposure to a specific stressor (i.e., endpoints never measured in the same study or not at all); and/or significant inconsistencies in empirical support across taxa and species that don't align with expected pattern for hypothesised AOP.**
MIE => KE1: **(Decrease, Coupling of OXPHOS leads to Decrease, ATP)**	**Empirical support of MIE => KE1 is** high. **Rationale:** The majority of the supporting studies covering several major types of uncouplers have shown good incidence, temporal and/or dose concordance in different organisms and cell types, with few cases of exceptions. For example, exposure of zebrafish (*Danio rerio*) embryos to 0.5 µM of the classical uncoupler 2,4-DNP led to significantly uncoupling of OXPHOS after 21h, whereas significant reduction ATP was only observed after 45h (Bestman 2015). In human colon cancer cells (SW480), exposure to 150 µM of the uncoupler flavanoid morin caused 60% reduction in MMP, whereas only around 35% decrease in ATP (Sithara 2017).	
KE1 => KE2: **(Decrease, ATP leads to Decrease, Cell proliferation)**	**Empirical support of KE1 => KE2 is** moderate. **Rationale:** Although only a few studies were found to be relevant, good temporal and incidence concordances were reported in zebrafish (Bestman 2015) and human cells (Sithara 2017).	

KE2 => KE3: **(Decrease, Cell proliferation leads to Decrease, Growth)**	**Empirical support of KE2 => KE3 is** low. **Rationale:** This KER was included in a very limited number of studies, as it addresses effects occurring at the apical level that *in vitro* studies cannot cover. There is one zebrafish study reporting concordant relationship between reduced cell proliferation and embryo growth with some inconsistencies (Bestman 2015).

Uncertainties, inconsistencies and critical gaps

There are several uncertainties related this AOP. **First**, although uncoupling of OXPHOS has been extensively investigated, no study has simultaneously included all KEs in the same analysis. Supporting evidence was summarized based on propagated information collected from multiple doses/concentrations, timepoints, cell types or species. This raises the concern about the overall relationship of the KEs in this AOP. **Second**, some evidence was generated using tumor cells. There is an uncertainty on whether these cells respond to uncouplers in a similar manner as normal cells. **Third**, full (i.e., >5 test doses/concentrations) dose-response analysis is lacking in most of the studies, leading to an uncertainty on the concordance across multiple doses and difficulties in deriving threshold values of perturbation such as point of departure (POD). **Fourth**, a few studies did not perform statistical analysis or clearly report the effect doses/concentrations. The authors were only able to estimate approximate values from the figures in their reports. **Fifth**, similarly to many other AOPs, the uncertainty increases with increasing the level of biological organization in the AOP. For example, growth inhibition at the apical level is likely not only attributed to impaired cell proliferation, but also other effects. This requires further development of the associated AOP network in the context of regulatory applications. **Sixth**, although the AOP is proposed for animals and plants in general, there is a significant lack of supporting data from invertebrates and plants. This calls for more follow-up studies using non-vertebrate models.

Some inconsistencies were also identified concerning the empirical support of the KERs. For example, a significant decrease followed by a significant increase of total ATP (KE1) was observed in human RD cells during a 48h exposure to the uncoupler FCCP (Kuruvilla 2003), possibly due to the enhancement of other ATP synthetic pathways (e.g., glycolysis) as a compensatory action to impaired OXPHOS (Jose 2011). In zebrafish embryos exposed to 2,4-DNP, significant growth inhibition (AO) was identified after 21h, whereas non-significant reductions in ATP (KE1) and cell proliferation (KE2) were reported (Bestman 2015). Chronic exposure of the plant *L. minor* to 3,5-DCP also led to more sensitive growth inhibition (AO) compared to uncoupling of OXPHOS (MIE). These inconsistencies are likely attributed to a combination of non-optimal sampling timepoints for analysis and potential compensatory mechanisms during chronic low-dose exposures.

There is currently no critical knowledge gap in this AOP. However, with the accumulation of experimental evidence and mechanistic understanding from a wider range of species, more intermediate KEs may be added to this AOP to reduce uncertainties and increase the predictability of this AOP.

Quantitative Consideration

Quantitative understanding of the KERs	**High**	**Change in $KE_{downstream}$ can be precisely predicted based on a relevant measure of $KE_{upstream}$. Uncertainty in the quantitative prediction can be precisely estimated from the variability in the relevant measure of $KE_{upstream}$. Known modulating factors and feedback/feedforward mechanisms are accounted for in the quantitative description. There is evidence that the quantitative relationship between the KEs generalises across the relevant applicability domain of the KER.**
	Moderate	**Change in $KE_{downstream}$ can be precisely predicted based on a relevant measure of $KE_{upstream}$. Uncertainty in the quantitative prediction is influenced by factors other than the variability in the relevant measure of $KE_{upstream}$. Quantitative description does not account for all known modulating factors and/or known feedback/feedforward mechanisms. The quantitative relationship has only been demonstrated for a subset of the overall applicability domain of the KER (e.g., based on a single species).**
	Low	**Only a qualitative or semi-quantitative prediction of the change in $KE_{downstream}$ can be determined from a measure of $KE_{upstream}$. Known modulating factors and/or known feedback/feedforward mechanisms are not accounted for. The quantitative relationship has only been demonstrated for a narrow subset of the overall applicability domain of the KER (e.g., based on a single species).**
MIE => KE1: **(Decrease, Coupling of OXPHOS leads to Decrease, ATP)**	**Quantitative understanding of MIE => KE1 is** high. **Rationale:** Multiple mathematical models have been developed for describing the quantitative relationships between uncoupling of OXPHOS and ATP synthesis in vertebrates (Beard 2005; Schmitz 2011; Heiske 2017; Kubo 2020). These models, however, are highly complex metabolic or systems biological models and warrant further simplification to be used for this AOP. In invertebrates, a regression based quantitative response-response relationship between uncoupling of OXPHOS and ATP depletion was proposed for the crustacean *Daphnia magna* under UVB stress (Song 2020).	

KE1 => KE2: **(Decrease, ATP leads to Decrease, Cell proliferation)**	**Quantitative understanding of KE1 => KE2 is** moderate. **Rationale:** Quantitative relationships between total ATP and cell proliferation have been extensively investigated (Ahmann 1987; Crouch 1993). In general, a monotonic positive relationship can be assumed for the two events, albeit the actual quantitative relationship can vary across biological systems (e.g. cell types and species). It has also been suggested that a threshold of ATP depletion (85-90% compared to normal status) may exist to determine whether proliferation arrest (<85-90%) or cell death (>85-90%) will be triggered in mammals (Nieminen 1994).
KE2 => KE3: **(Decrease, Cell proliferation leads to Decrease, Growth)**	**Quantitative understanding of KE2 => KE3 is** moderate. **Rationale:** Multiple mathematical models describing the quantitative relationships between cell proliferation and tissue growth exist for both animals (Binder 2008) and plants (Mosca 2018). There are also numerous models that are specifically developed for predicting tumor growth based on the proliferation rate (Jarrett 2018). However, there is currently a lack of quantitative model to link cell proliferation and individual growth in the presence of uncouplers.

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Appendix 1

List of MIEs in this AOP

Event: 1446: Decrease, Coupling of oxidative phosphorylation (https://aopwiki.org/events/1446)

Short Name: Decrease, Coupling of OXPHOS

Key Event Component

Process	Object	Action
oxidative phosphorylation uncoupler activity		increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:267 - Uncoupling of oxidative phosphorylation leading to growth inhibition (5) (https://aopwiki.org/aops/267)	MolecularInitiatingEvent
Aop:263 - Uncoupling of oxidative phosphorylation leading to growth inhibition (1) (https://aopwiki.org/aops/263)	MolecularInitiatingEvent
Aop:264 - Uncoupling of oxidative phosphorylation leading to growth inhibition (2) (https://aopwiki.org/aops/264)	MolecularInitiatingEvent
Aop:265 - Uncoupling of oxidative phosphorylation leading to growth inhibition (3) (https://aopwiki.org/aops/265)	MolecularInitiatingEvent
Aop:266 - Uncoupling of oxidative phosphorylation leading to growth inhibition (4) (https://aopwiki.org/aops/266)	MolecularInitiatingEvent
Aop:268 - Uncoupling of oxidative phosphorylation leading to growth inhibition (6) (https://aopwiki.org/aops/268)	MolecularInitiatingEvent

Stressors

Name
2,4-Dinitrophenol
Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone
Carbonyl cyanide m-chlorophenyl hydrazone
Pentachlorophenol
Triclosan

Name
Emodin
Malonoben

Biological Context

Level of Biological Organization
Molecular

Cell term

Cell term
cell

Evidence for Perturbation by Stressor

Overview for Molecular Initiating Event

Decreased coupling of oxidative phosphorylation can be directly triggered by “uncouplers” as a molecular initiating event. Most of the chemical uncouplers are protonophores, a type of proton binders that can translocate protons across membranes. These protonophores several common structural characteristics, such as bulky hydrophobic moiety, an acid dissociable group and a strong electron-withdrawing group (Terada 1990). Weak acids such as phenols, benzimidazoles and salicylic acids are considered potential protonophores. Classical uncouplers, such as carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP), carbonyl cyanide m-chlorophenyl hydrazone (CCCP), 2,4-dinitrophenol (DNP), pentachlorophenol (PCP) and SF-6847 (Terada 1990), as well as new uncouplers, such as triclosan (Shim 2016; Weatherly 2016), emodin (Sugiyama 2019), and hydroxylated polybrominated diphenyl ethers (PBDEs) (Legradi 2014) have been widely investigated in vertebrates. Computational predictions based on quantitative structure-activity relationships (Russom 1997; Schultz 1997; Naven 2012; Dreier 2019; Troger 2020) and in vitro high-throughput screening (Escher 2002; Attene-Ramos 2013; Attene-Ramos 2015; Xia 2018) have facilitated the identification and classification of potential uncouplers from a massive list of chemicals.

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
zebrafish	Danio rerio	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=7955)
human	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
mouse	Mus musculus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)
rat	Rattus norvegicus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)
Lemna minor	Lemna minor	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=4472)

Life Stage Applicability

Life Stage	Evidence
Embryo	High
Juvenile	High

Life Stage	Evidence
Adult, reproductively mature	Moderate

Sex Applicability

Sex	Evidence
Unspecific	High

Taxonomic applicability domain

This key event is in general considered applicable to most eukaryotes, as the mitochondrion and oxidative phosphorylation are highly conserved (Roger 2017).

Life stage applicability domain

This key event is considered applicable to all life stages, as ATP synthesis by oxidative phosphorylation is an essential biological process for most living organisms.

Sex applicability domain

This key event is considered sex-unspecific, as both males and females use oxidative phosphorylation as a main process to generate ATP.

Key Event Description

Decreased coupling of oxidative phosphorylation (OXPHOS), or uncoupling of OXPHOS, describes dissipation of protonmotive force (PMF) across the inner mitochondrial membrane (IMM) by environmental stressors. In eukaryotes, the mitochondrial electron transport chain mediates a series of redox reactions to create a PMF across the IMM. The PMF is used as energy to drive adenosine triphosphate (ATP) synthesis through phosphorylation of adenosine diphosphate (ADP). These processes are coupled and referred to as OXPHOS. A number of chemicals can dissipate the PMF, leading to uncoupling of OXPHOS. This key event describes the main outcome of the interactions between an uncoupler and the transmembrane PMF. An uncoupler can bind to a proton in the mitochondrial inter membrane space, transport the proton to the matrix side of the IMM, release the proton and move back to the inter membrane space. These processes are repeated until the transmembrane PMF is dissipated. This KE is therefore a lumped term of these processes and represents the final consequence of the interactions.

How it is Measured or Detected

Uncoupling of oxidative phosphorylation can be indicated by reduced mitochondrial membrane potential, increased proton leak and/or increased oxygen consumption rate. Mitochondrial membrane potential can be measured using fluorescent probes such as TMRM (tetramethylrhodamine, methyl ester, perchlorate), TMRE (tetramethylrhodamine, ethyl ester, perchlorate) and JC-1 (Perry 2011). Proton leak and oxygen consumption rate can be measured using a high-resolution respirometry (Affourtit 2018) or a Seahorse XF analyzer (Divakaruni 2014).

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List of Key Events in the AOP

Event: 1771: Decrease, Adenosine triphosphate (https://aopwiki.org/events/1771)

Short Name: Decrease, ATP

Key Event Component

Process	Object	Action
ATP biosynthetic process	ATP	decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:328 - Excessive reactive oxygen species production leading to mortality (2) (https://aopwiki.org/aops/328)	KeyEvent
Aop:329 - Excessive reactive oxygen species production leading to mortality (3) (https://aopwiki.org/aops/329)	KeyEvent
Aop:264 - Uncoupling of oxidative phosphorylation leading to growth inhibition (2) (https://aopwiki.org/aops/264)	KeyEvent
Aop:263 - Uncoupling of oxidative phosphorylation leading to growth inhibition (1) (https://aopwiki.org/aops/263)	KeyEvent
Aop:299 - Excessive reactive oxygen species production leading to population decline via reduced fatty acid beta-oxidation (https://aopwiki.org/aops/299)	KeyEvent
Aop:311 - Excessive reactive oxygen species production leading to population decline via mitochondrial dysfunction (https://aopwiki.org/aops/311)	KeyEvent

Stressors

Name
Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone
Carbonyl cyanide m-chlorophenyl hydrazone
2,4-Dinitrophenol
Malonoben
Pentachlorophenol
Triclosan
Emodin

Biological Context

Level of Biological Organization
Cellular

Cell term

Cell term
cell

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
zebrafish	Danio rerio	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=7955)
human	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
rat	Rattus norvegicus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)
mouse	Mus musculus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)

Life Stage Applicability

Life Stage	Evidence
Embryo	High
Juvenile	High
Adult, reproductively mature	Moderate

Sex Applicability

Sex	Evidence
Unspecific	High

Taxonomic applicability domain

This key event is in general considered applicable to all eukaryotes utilizing ATP as a direct source of energy and signaling molecule.

Life stage applicability domain

This key event is considered applicable to all life stages, as all developmental stages require energy supply to maintain necessary physiological processes.

Sex applicability domain

This key event is considered sex-unspecific, as both males and females use ATP as an essential energy molecule.

Key Event Description

Decreased adenosine triphosphate (ATP) describes the loss of balance between ATP synthesis and ATP consumption, leading to reduced total ATP. As a primary form of biological energy, ATP is used by many biological processes (Bonora 2012). Decrease in ATP level normally attributes to metabolic disorders in major ATP synthetic pathways, such as mitochondrial oxidative phosphorylation, fatty acid β-oxidation, glycolysis and plant photophosphorylation.

How it is Measured or Detected

The ATP level in cells or tissue can be quantified using a well-established ATP bioluminescent assay (Lemasters 1978; Wibom 1990). Assay principles: ATP can react with luciferase and luciferin from firefly and the luminescence emitted from the reaction is proportional to the ATP concentration:

ATP + D-Luciferin + O_2 è Oxyluciferin + AMP + PPi + CO_2 + Light

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Event: 1821: Decrease, Cell proliferation (https://aopwiki.org/events/1821)

Short Name: Decrease, Cell proliferation

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:263 - Uncoupling of oxidative phosphorylation leading to growth inhibition (1) (https://aopwiki.org/aops/263)	KeyEvent

Stressors

Name
2,4-Dinitrophenol
Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone
Carbonyl cyanide m-chlorophenyl hydrazone
Pentachlorophenol
Triclosan
Emodin
Malonoben

Biological Context

Level of Biological Organization
Cellular

Cell term

Cell term
cell

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
zebrafish	Danio rerio	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=7955)
human	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
rat	Rattus norvegicus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)
mouse	Mus musculus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)

Life Stage Applicability

Life Stage	Evidence
Embryo	High
Juvenile	High

Sex Applicability

Sex	Evidence
Unspecific	High

Taxonomic applicability domain

This key event is in general applicable to all eukaryotes.

Life stage applicability domain

This key event is in general applicable to all life stages.

Sex applicability domain

This key event is sex-unspecific.

Key Event Description

Decreased cell proliferation describes the outcome of reduced cell division and cell growth. Cell proliferation is considered the main mechanism of tissue and organismal growth (Conlon 1999). Decreased cell proliferation has been associated with abnormal growth-factor signaling and cellular energy depletion (DeBerardinis 2008).

How it is Measured or Detected

Cell proliferation can be measured using the well-established 5-bromo-2'-deoxyuridine (BrdU) (Raza 1985; Muir 1990) or 5-ethynyl-2´-deoxyuridine (EdU) assay. Both methods measure DNA synthesis in dividing cells to indicate proliferation status. The assay kits are commercially available and have high-throughput formats.

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List of Adverse Outcomes in this AOP

Event: 1521: Decrease, Growth (https://aopwiki.org/events/1521)

Short Name: Decrease, Growth

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:263 - Uncoupling of oxidative phosphorylation leading to growth inhibition (1) (https://aopwiki.org/aops/263)	AdverseOutcome
Aop:264 - Uncoupling of oxidative phosphorylation leading to growth inhibition (2) (https://aopwiki.org/aops/264)	AdverseOutcome
Aop:265 - Uncoupling of oxidative phosphorylation leading to growth inhibition (3) (https://aopwiki.org/aops/265)	AdverseOutcome
Aop:266 - Uncoupling of oxidative phosphorylation leading to growth inhibition (4) (https://aopwiki.org/aops/266)	AdverseOutcome
Aop:267 - Uncoupling of oxidative phosphorylation leading to growth inhibition (5) (https://aopwiki.org/aops/267)	AdverseOutcome
Aop:268 - Uncoupling of oxidative phosphorylation leading to growth inhibition (6) (https://aopwiki.org/aops/268)	AdverseOutcome

Stressors

Name
2,4-Dinitrophenol
Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone
Carbonyl cyanide m-chlorophenyl hydrazone
Pentachlorophenol
Triclosan
Emodin
Malonoben

Biological Context

Level of Biological Organization
Individual

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	Moderate	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
rat	Rattus norvegicus	Moderate	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)
mouse	Mus musculus	Moderate	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)
zebrafish	Danio rerio	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=7955)
fathead minnow	Pimephales promelas	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=90988)
Lemna minor	Lemna minor	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=4472)
Daphnia magna	Daphnia magna	Moderate	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=35525)

Life Stage Applicability

Life Stage	Evidence
Embryo	High
Juvenile	High

Sex Applicability

Sex	Evidence
Unspecific	High

Taxonomic applicability domain

This key event is in general applicable to all eukaryotes.

Life stage applicability domain

This key event is applicable to early life stages such as embryo and juvenile.

Sex applicability domain

This key event is sex-unspecific.

Key Event Description

Decreased growth refers to a reduction in size and/or weight of a tissue, organ or individual organism. Growth is normally controlled by growth factors and mainly achieved through cell proliferation (Conlon 1999).

How it is Measured or Detected

Growth can be indicated by measuring weight, length, total volume, and/or total area of a tissue, organ or individual organism.

Regulatory Significance of the AO

Growth is a regulatory relevant chronic toxicity endpoint for almost all organisms. Multiple OECD test guidelines have included growth either as a main endpoint of concern, or as an additional endpoint to be considered in the toxicity assessments. Relevant test guidelines include, but not only limited to:

-Test No. 208: Terrestrial Plant Test: Seedling Emergence and Seedling Growth Test

-Test No. 212: Fish, Short-term Toxicity Test on Embryo and Sac-Fry Stages

-Test No. 215: Fish, Juvenile Growth Test

-Test No. 241: The Larval Amphibian Growth and Development Assay (LAGDA)

-Test No. 201: Freshwater Alga and Cyanobacteria, Growth Inhibition Test

-Test No. 221: Lemna sp. Growth Inhibition Test

-Test No. 228: Determination of Developmental Toxicity to Dipteran Dung Flies (Scathophaga stercoraria L. (Scathophagidae), Musca autumnalis De Geer (Muscidae))

-Test No. 211: Daphnia magna Reproduction Test

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Appendix 2

List of Key Event Relationships in the AOP

List of Adjacent Key Event Relationships

Relationship: 2203: Decrease, Coupling of OXPHOS leads to Decrease, ATP (https://aopwiki.org/relationships/2203)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Uncoupling of oxidative phosphorylation leading to growth inhibition (1) (https://aopwiki.org/aops/263)	**adjacent**	**High**	**High**

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
zebrafish	Danio rerio	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=7955)
human	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
rat	Rattus norvegicus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)
mouse	Mus musculus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)

Life Stage Applicability

Life Stage	Evidence
Embryo	High
Juvenile	High

Sex Applicability

Sex	Evidence
Unspecific	High

Mitochondrial oxidative phosphorylation and ATP synthesis are highly conserved biological processes in eukaryotes. Uncoupling of OXPHOS leading to ATP depletion have been well documented for human, rodents and fish (see discussion above).

Key Event Relationship Description

This key event relationship describes the dissipation of protonmotive force across the inner mitochondrial membrane by uncouplers (uncoupling of oxidative phosphorylation), leading to reduced total adenosine triphosphate (ATP) pool in cells or organisms.

Evidence Supporting this KER

The evidence supporting this KER is high.

Biological Plausibility
The biological plausibility of this KER is considered high.

Rationale: In eukaryotic cells, the major metabolic pathways responsible for ATP production are OXPHOS, citric acid (TCA) cycle, glycolysis and photosynthesis. Oxidative phosphorylation is much (theoretically 15-18 times) more efficient than the rest due to high energy derived from oxygen during aerobic respiration (Schmidt-Rohr 2020). As the ATP level is relatively balanced between production and consumption (Bonora 2012), ATP depletion is a plausible consequence of reduced ATP synthetic efficiency following uncoupling of OXPHOS.

Empirical Evidence
The empirical support of this KER is considered high.

Rationale: The majority of the supporting studies show good incidence, temporal and/or dose concordance in different organisms and cell types after exposure to known uncouplers, with few cases of exceptions.

- Exposure of zebrafish (Danio rerio) embryos to 0.5 µM of the classical uncoupler 2,4-DNP led to significantly uncoupling of OXPHOS after 21h, whereas significant reduction in ATP was only observed after 45h (Bestman 2015).
- In human colon cancer cells (SW480), exposure to 150 µM of the uncoupler flavanoid morin caused 60% reduction in MMP, whereas only around 35% decrease in ATP (Sithara 2017).

Uncertainties and Inconsistencies

- A significant decrease followed by a significant increase of total ATP was observed in human RD cells during a 48h exposure to the uncoupler FCCP (Kuruvilla 2003), possibly due to the enhancement of other ATP synthetic pathways (e.g., glycolysis) as a compensatory action to impaired OXPHOS (Jose 2011).
- In zebrafish embryos exposed to 2,4-DNP, significant growth inhibition (AO) was identified after 21h, whereas non-significant reductions in ATP (KE1) and cell proliferation (KE2) were reported (Bestman 2015).
- Chronic exposure of the plant L. minor to 3,5-DCP also led to more sensitive growth inhibition (AO) compared to uncoupling of OXPHOS (MIE).

Quantitative Understanding of the Linkage

The quantitative understanding of this KER is high.

Rationale: Multiple mathematical models have been developed for describing the quantitative relationships between uncoupling of OXPHOS and ATP synthesis in vertebrates (Beard 2005; Schmitz 2011; Heiske 2017; Kubo 2020). These models, however, are highly complex metabolic or systems biological models and warrant further simplification to be used for this AOP.

Response-response relationship
A regression based quantitative response-response relationship between uncoupling of OXPHOS and ATP depletion was proposed for the crustacean *Daphnia magna* under UVB stress (Song 2020).

References

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Schmidt-Rohr K. 2020. Oxygen is the high-energy molecule powering complex multicellular life: fundamental corrections to traditional bioenergetics. *ACS Omega* 5:2221-2233. DOI: 10.1021/acsomega.9b03352.

Schmitz JPJ, Vanlier J, van Riel NAW, Jeneson JAL. 2011. Computational modeling of mitochondrial energy transduction. 39:363-377. DOI: 10.1615/CritRevBiomedEng.v39.i5.20.

Sithara T, Arun KB, Syama HP, Reshmitha TR, Nisha P. 2017. Morin inhibits proliferation of SW480 colorectal cancer cells by inducing apoptosis mediated by reactive oxygen species formation and uncoupling of Warburg effect. *Frontiers in Pharmacology* 8. DOI: 10.3389/fphar.2017.00640.

Song Y, Xie L, Lee Y, Tollefsen KE. 2020. De novo development of a quantitative adverse outcome pathway (qAOP) network for ultraviolet B (UVB) radiation using targeted laboratory tests and automated data mining. *Environmental Science & Technology* 54:13147-13156. DOI: 10.1021/acs.est.0c03794.

Relationship: 2204: Decrease, ATP leads to Decrease, Cell proliferation (https://aopwiki.org/relationships/2204)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Uncoupling of oxidative phosphorylation leading to growth inhibition (1) (https://aopwiki.org/aops/263)	**adjacent**	**Moderate**	**Moderate**

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
zebrafish	Danio rerio	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=7955)
human	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)

Life Stage Applicability

Life Stage	Evidence
Embryo	High

Sex Applicability

Sex	Evidence
Unspecific	High

Multiple studies have reported this relationship in zebrafish embryos and mammlian cells after exposure to chemicals affecting ATP synthesis.

Key Event Relationship Description

This key event relationship describes reduced adenosine triphosphate (ATP) supply leading to reduced cell proliferation (cell growth, division or a combination of these).

Evidence Supporting this KER

The evidence supporting this KER is moderate.

Biological Plausibility
The biological plausibility of this KER is considered high.

Rationale: Cell proliferation is a well-known ATP-dependent process. Cell division processes, such as the mitotic cell cycle uses ATP for chromosome movements and DNA replication (Kingston 1999). The synthetic processes of major cellular components that are necessary for cell structure and growth, such as proteins and lipids, also require sufficient ATP supply (Bonora 2012). Depletion of ATP therefore has a negative

impact on these processes.

Empirical Evidence
The empirical support of this KER is considered moderate.

Rationale: Although only a few studies were found to be relevant, good temporal and incidence concordances were reported in zebrafish (Bestman 2015) and mammalian cells (Sithara 2017).

References

Ahmann FR, Garewal HS, Schifman R, Celniker A, Rodney S. 1987. Intracellular adenosine triphosphate as a measure of human tumor cell viability and drug modulated growth. *In Vitro Cellular & Developmental Biology* 23:474-480. DOI: 10.1007/BF02628417.

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Bonora M, Patergnani S, Rimessi A, De Marchi E, Suski JM, Bononi A, Giorgi C, Marchi S, Missiroli S, Poletti F, Wieckowski MR, Pinton P. 2012. ATP synthesis and storage. *Purinergic Signalling* 8:343-357. DOI: 10.1007/s11302-012-9305-8.

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Relationship: 2205: Decrease, Cell proliferation leads to Decrease, Growth (https://aopwiki.org/relationships/2205)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Uncoupling of oxidative phosphorylation leading to growth inhibition (1) (https://aopwiki.org/aops/263)	**adjacent**	**Moderate**	**Moderate**

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
zebrafish	Danio rerio	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=7955)

Life Stage Applicability

Life Stage	Evidence
Embryo	High

Sex Applicability

Sex	Evidence
Unspecific	High

There is one study reporting this relationship in zebrafish embryos.

Key Event Relationship Description

This key event relationship describes reduced cell proliferation (cell growth, division or a combination of these) leading to reduced tissue, organ or individual growth.

Evidence Supporting this KER

The evidence supporting this KER is moderate.

Biological Plausibility
The biological plausibility of this KER is considered high.

Rationale: The biological causality between cell proliferation and growth has also been well established. It is commonly accepted that the size of an organism, organ or tissue is dependent on the total number and volume of the cells it contains, and the amount of extracellular matrix and fluids (Conlon 1999). Impairment to cell proliferation can logically affect tissue and organismal growth.

Empirical Evidence
The empirical support of this KER is considered low.

Rationale: This KER was included in a very limited number of studies, as it addresses effects occurring at the apical level that in vitro studies cannot cover. There is one zebrafish study reporting concordant relationship between reduced cell proliferation and embryo growth with some inconsistencies (Bestman 2015).

References

Bestman JE, Stackley KD, Rahn JJ, Williamson TJ, Chan SS. 2015. The cellular and molecular progression of mitochondrial dysfunction induced by 2,4-dinitrophenol in developing zebrafish embryos. *Differentiation* 89:51-69. DOI: 10.1016/j.diff.2015.01.001.

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