**Internal review charge questions - February 2018**

## AOP Information

* AOP title: Histone deacetylase inhibition leading to testicular toxicity
* Author: Shihori Tanabe, Akihiko Hirose, Takashi Yamada ([t-yamada@nihs.go.jp](mailto:t-yamada@nihs.go.jp))
* Associated wiki page: <https://aopwiki.org/aops/212>

## Reviewers

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#### Date review completed: 04/25/2018

## Review

**Section 1:**

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| **AOP identifier/Title**  *Does the name of the AOP follow the right convention (MIE or first KE leading to AO)?*  *Does the name of the AOP reflect its content/domain?* |

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| **Reviewers' responses and comments**  **PR:** Yes,broadly speaking the AOP title follows the guidance and reflects its content.  **SR1:** Yes  **SR2:** The name of this AOP “Histone deacetylase inhibition leading to testicular toxicity” follows the right convention and reflects the content. |

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| **Author response:**  Thank you very much for the agreement. |

**Section 2:**

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| **Authors**  *Is it clear who the authors/developers of the AOP are?*  *Contact information for one or more corresponding author(s) should be included.* |

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| **Reviewers' responses and comments**  **PR:** Yes, the authors identity and affiliation information is provided. The point of contact and contributors are clearly noted.  **SR1:** Yes. Shihori Tanabe, Akihiko Hirose, Takashi Yamada. From the Division of Risk Assessment, Biological Safety Research Center, National Institute of Health Sciences  **SR2:** Three authors are listed for this AOP. The contact is mentioned in the web site version of this AOP (Takashi Yamada). Two contributors are mentioned: Shihori Tanabe and Takashi Yamada. |

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| **Author response:**  Thank you very much. |

**Section 3:**

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| **Date of updating**  *Reviewer should indicate the date stamp on the PDF snapshot under review.* |

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| **Reviewers' responses and comments**  **PR:** Reviewing the content in the AOP-Wiki, last modified January 30, 2018, 20:54  **SR1:** Indicated as: 2018-02-23 15:17  **SR2:** thesnapshot used for this review is from February, 23rd 2018 |

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| **Author response:**  Thank you very much. |

**Section 4:**

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| **Abstract**  *Does the abstract concisely describe the main content of the AOP?* |

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| **Reviewers' responses and comments**  **PR:** Yes. The abstract describes the main content and relevance of the AOP. It could probably benefit from some light editing to improve clarity.  **SR1:** The abstract is great. However, I have some comments regarding the title/content of the Adverse Outcome. Please address these comments in the Adverse Outcome section, and adjust the abstract accordingly, if necessary.  **SR2:** The abstracts summarizes the content of the AOP: from MIE via key events to AO. Model stressors are also described here. It may help to have a list of the abbreviations used frm this section, especially regarding the stressors. The authors provide the rational for developing this AOP. It may be useful to add a couple of sentences on the overall supporting evidence and gaps/challenges for this AOP. |

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| **Author response:**  In the abstract, the sentences were added for the overall supporting evidence and gaps/challenges for this AOP. According to the reviewers’ comments, AO (spermatocyte depletion, testis atrophy/weght loss) in the previous version of AOP was divided into KE and AO, and the description about AO was up-dated. The abbreviation list was created for this section. |

**Section 5:**

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| **Molecular Initiating Event**  *Is a MIE described? If yes, then:*  *Is the MIE description clear and is it biologically plausible?*  *Is the MIE described in a way that allows its use in other AOPs?*  *Are measurement/prediction methods specified and adequately described/referenced?*  *Is the biological context (inc. taxonomic applicability/relevance, level of biological organisation) specified and explained sufficiently?*  *Have chemical initiators (prototypical chemicals or chemical features) been identified?* |

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| **Reviewers' responses and comments**  **PR:** The MIE is Event 1502. The MIE description is relatively clear and biologically plausible. See comment below regarding measurement method. The taxonomic applicability is defined quite narrowly based on where clear evidence exists. The authors might consider also defining the expected domain of applicability based on the conservation of HDAC as a target. Life stage and sex applicability of the MIE have not been defined. Those should be added if possible. A number of chemical initiators were identified using both structured chemical name fields and free text.   * Event component terms should be added. * Structured annotation terms for life stage and sex applicability should be selected if possible. * The authors suggest it is measured/detected by measuring a decrease in histone acetylation. This seems both counter-intuitive and directly contradicts the next KE in the pathway which is an increase in histone acetylation. It also begs the question of whether this MIE can be measured independent of event 1503. Even if it can’t be measured directly, I guess I would favor still including this MIE, as one could conceivably used structure based approaches to identify chemicals likely to directly inhibit HDAC. This make a cleaner connection to a chemical category than the general measurement of histone acetylation, but if there are additional methods for more directly measuring the activity of HDAC, it would be nice to cite those. * Please provide both first author and year information when citing references.   **SR1: Is the MIE description clear and is it biologically plausible?**  Yes. An excellent and concise description.  **Is the MIE described in a way that allows its use in other AOPs?**  Cuurently, the MIE describes its “site of action” as the spermatocytes in testis. This severely limits the ability of this KE (MIE) to be used in other AOPs. Presumably, HDAC has a similar function in many (all?) cell types. In fact, the authors do describe the various tissues in which HDACs are found. I recommend removing the spermatocyte specificity from the description to allow more athors to borrow this very useful MIE.  **Are measurement/prediction methods specified and adequately described/referenced?**  While the methods appear to be adequately described and referenced… the methods for measuring HDI seem to be the same as the methods for measuring the first KE (increased histone acetylation). Are there any “more direct” methods to measure the activity of the enzyme? Otherwise…. Perhaps the MIE and KE1 might be considered the same thing…  **Is the biological context (inc. taxonomic applicability/relevance, level of biological organisation) specified and explained sufficiently?**  Presumably, HDAC inhibition is relevent to all eukarytic organisms? Perhaps the authors can indicate in the text section that HDI is relevant to most species, but there is very strong experimental evidence in human, rat, and mouse models.  The handbook does indeed state that “in many cases, individual species identified in these structured fields will be those for which the strongest evidence used in constructing the AOP was available in relation to this KE.” So I do believe that the authors have completed this section correctly.  However, this may be an issue that may need to be addressed by the EAGMST: I know it is not the intention… but adding specific species into this section may inadvertently preclude others when “searching/filtering” though the AOP-KB.  **Have chemical initiators (prototypical chemicals or chemical features) been identified?**  Evidence for chemical initators appears well documented and referenced.  **SR2:** The MIE is well described here and is clearly biologically plausible. The authors provide information regarding stressors, how to measure the MIE and the biological context. Its is usable for other AOPs. |

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| **Author response:**  Thank you very much for valuable comments. According to the reviewers’ comments, the manuscript was revised as follows:  Key event components were added.  Structured life stages and sex applicability were added.  The method directly measuring the HDAC activity using UHPLC-ESI-MS/MS (Zwick V et al, 2016) was cited. In this method, the ratio of deacetylated peptide to acetylated peptide is calculated.  The citations of the reference were updated to include both the first author and year.  The spermatocyte specificity was removed to allow other authors to cite this MIE.  The description about the domain of applicability was revised to indicate that HDAC inhibition is relevant to most species, but there are relatively abundant experimental evidences in human, rat and mouse models. |

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| Key Events *Are the KE descriptions clear on how the events work and are they biologically plausible?*  *Are the KEs described in a way that allows their reuse in other AOPs?*  *Are measurement methods specified and adequately described/referenced?*  *Is the biological context (inc. taxonomic applicability/relevance, level of biological organisation) specified and explained sufficiently?* |

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| **Reviewers' responses and comments**  **PR:** Specific comments are provided for each KE. Comments that apply to all KEs are listed as “All”   * All – Structured ontology terms defining the Key Event Components should be selected. * All – structure terms for life stage, sex, and taxonomic applicability should be selected if possible. The authors cite taxa for which specific evidence of KE applicability is known. At least in the narrative, it would also be useful to identify the probable domain of applicability based on conservation. * Event 1503 – description is adequate and plausible. Measurement approaches are specified. More details regarding the domain of applicability would be helpful. * Event 1504 – description is adequate and plausible. Measurement approaches are specified. More details regarding biological context could be added. * Event 1505. Rather than focusing on a description of cell cycle, the current description makes reference to upstream and downstream KEs and their connection to cell cycle. This may hinder the ability to use this KE description for other AOPs. Consider focusing the description more narrowly on cell cycle. The measurement methods (flow cytometry, FACS, live cell imaging) should be described without specific to HDAC inhibition. See general comments on domain of applicability. * Event 1262. Description makes reference to both specific chemicals and other stressors that may cause apoptosis rather than simply describing what apoptosis is. Consequently, this event is not written in a particularly modular fashion and confounds content that should go in the KER descriptions with more general description of the biology that should be provided in the KE description. Relative to the methods, it is not clear how the last two sentences regarding proliferative/viability of NHDFs or proliferation of HDAC-/- cells represent a specific measure of apoptosis. More explanation is needed. See general comments regarding domain of applicability.   **SR1: KE1: 1503 - Histone acetylation, increase**  **Are the KE descriptions clear on how the events work and are they biologically plausible?**  Excellent, plausible and concise description with adequate references  **Are the KEs described in a way that allows their reuse in other AOPs?**  Yes.This is a great example of a well written “portable” KE.  **Are measurement methods specified and adequately described/referenced?**  Please see my comments regarding the methods described in the MIE. It appears that this KE and the MIE have the same measurement methods… leading one to ask: In practice, do these two KEs represent the same thing?  **Is the biological context (inc. taxonomic applicability/relevance, level of biological organisation) specified and explained sufficiently?**  Yes. But again, I recommend that the authors indicate that this KE is relevent to a much broader range of cells/tisses/species (all eukarytic cells?)  **KE2: 1504-p21 expression, increase**  **Are the KE descriptions clear on how the events work and are they biologically plausible?**  Excellent, plausible and concise description. The reference to valproic acid is proably not necessary, since this is chemical specific information. Alternatively, the authors could include a general description that many types of chemicals that can lead to altered p21 expression.  Minor: Can the event title also include the genes alternate name (CDKN1A)? I believe this is the accepted name in GenBank.  **Are the KEs described in a way that allows their reuse in other AOPs?**  Yes. But see biological context section below.  **Are measurement methods specified and adequately described/referenced?**  Adequately described and refernced.  **Is the biological context (inc. taxonomic applicability/relevance, level of biological organisation) specified and explained sufficiently?**  The examples provided are very specific to this AOP (all HDAC inhibitors). This KE is much more broadly applicable than this. Again, a more general description of the domain of applicability would me more approriate.  **KE3: 1505- cell cycle disorder**  **Are the KE descriptions clear on how the events work and are they biologically plausible?**  Regarding the title: suggest “cell cycle, abnormal” or “cell cycle, disrupted”  The overal description is too spermatocyte-specific and HDI-specific. Cell cycle disruption can be a very common key event in many cell from a variety of treatments. Please make the description more general so that it can be borrowed by other AOPs.  **Are the KEs described in a way that allows their reuse in other AOPs?**  See above comment  **Are measurement methods specified and adequately described/referenced?**  The reference to the HDI inhibitor is not necessary. Otherwise appears to be adequate  **Is the biological context (inc. taxonomic applicability/relevance, level of biological organisation) specified and explained sufficiently?**  Again, a more general decription of the domain of applicability would me more appropriate, since this KE should be relevent to all replicating cells  **KE4: 1262- Apoptosis**  GENERAL: There are already very many KE events for apoptosis described in the wiki. I recommend that the authors borrow an existing KE an/or co-ordinate with the other authors of one general apoptosis KE that can be shared by many AOPs.  Alternatively, another option is to make the KE more specific to spermatocytes. However, this should only be done if there are characteristics of apoptosis that are UNIQUE in spermatocytes…. Otherwise the more general/shared KE option is preferred.  **SR2:** Similarly to the MIE, the Kes are well described. It complies with the AOP convention. Cell cycle disorders and apoptosis are generic processes. Any possibility to be more specific for this AOP? Especially given the fact that the authors raised the possibility for a role played by P53 and/or NFKB in the apoptosis KE. |

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| **Author response:**  For PR:  The Key Event Components were added for all key events. Structured ontology terms defining the Key Event Components and structure terms for life stage, sex, and taxonomic applicability were selected in case by case. The probable domain of applicability was added in the narrative in case appropriate.  In Event 1505, the KE description was focused more narrowly on cell cycle. The measurement methods (flow cytometry, FACS, live cell imaging) was described without specific to HDAC inhibition.  In Event 1262, the description was revised to simply describe what apoptosis is. The last two sentences regarding proliferative/viability of NHDFs or proliferation of HDAC-/- cells were revised to explain more in detail.  The structured domain of applicability tables in the free-text domain of applicability section was changed to be narrative.  For SR1:  The KE1:1503 was indicated to be relevant to a broader range of cells/tissues/species.  The KE2:1504, the reference to valproic acid was deleted and the general description that many types of chemicals lead to altered p21 expression was added. The alternate official name of p21 was added to the event title as p21 (CDKN1A) expression, increase. The examples specific to this AOP were revised to describe the domain of applicability more generally.  The KE3:1505, the title was changed to “cell cycle, disrupted.” The overall description which is specific to spermatocyte and HDI was revised to be more general so that it can be borrowed by other AOPs. The reference to the HDI inhibitor was deleted. The domain of applicability was revised to be more general and indicate that is relevant to all replicating cells.  The KE4:1262, the Key Event for apoptosis may be linked to the existing KE describing apoptosis induction such as KE1365. It seems that KE1365 is not fulfilled yet and needed to be discussed. The description about the possibility for a role played by p53 and/or NFkappaB in the apoptosis were added to the KE4:1262 apoptosis. |

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| **Adverse Outcome**  *Is an AO described? If yes, then:*  *Is the AO description clear and is it biologically plausible?*  *Is the AO described in a way that allows its use in other AOPs?*  *Are measurement methods specified and adequately described/referenced?*  *Is the biological context (inc. taxonomic applicability/relevance, level of biological organisation) specified and explained sufficiently?*  *Has the regulatory relevance of the AO been described?* |

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| **Reviewers' responses and comments**   * **PR:** Event 1506 is the adverse outcome.   + The event title could probably just be “testicular toxicity” with spermatocyte depletion and testis atrophy/weight loss included as ways that testicular toxicity is measured.   + This KE description should not include reference to the effects of specific chemicals on sperm production.   + This KE is currently not described in a way that allows for/facilitates use in other AOPs.   + The text under how it is measured or detected appears to perhaps be cut and pasted directly from other papers in which these measurements were made. This section should be simplified. For example, state that testicular toxicity can be measured through documentation of spermatocyte depletion and/or testis atrophy/weight loss. Then describe generally how each of those are measured. Reference to specific chemicals, fixatives, reagents, etc. is probably too detailed. Readers can seek out the cited papers to find all those details. This is a case where too much information makes it difficult to use.   + Similarly, the domain of applicability statement should be much more clear and concise – this KE is applicable to these species, because……. – cite supporting literature.   + Regulatory significance of the AO is not addressed.   **SR1: Is the AO description clear and is it biologically plausible?**  Title: spermatocyte depletion, testis atrophy/weight loss (testicular toxicity)  The title seems like it could be multiple KEs … I suggest reducing it to “spermatocyte depletion”  Additionally, shouldn’t the ultimate AO be “reduced sperm count”? or “reduced fertility”? Or even “population decline”? I suggest adding at least “reduced sperm count” to the AOP.  Another option (which may have been authors original intention) is to change the title to be more general and simple call it “testicular toxicity”.  Personally, I prefer to option of parsing it into separete discretely measurable KEs.  **Is the AO described in a way that allows its use in other AOPs?**  The descritpion is a bit too specific to HDIs. Please make the desciption more general so that it can be borrowed by other AOPs.  **Are measurement methods specified and adequately described/referenced?**  There is too much information in the methods section. It describes specific experiments and describes methods are not very specifc to this endpoint:  For example, the authors included the following methods:   1. Sperm count by haemocytometer (measures sperm… not spermatocytes) 2. Weight fo testes 3. TUNEL stain (stain apoptotic cells…the previous KE) 4. Flow cytometry (probably one the most appropriate/direct methods) 5. Microscope (probably one the most appropriate/direct methods) 6. determination of total LDH and LDH-X   Please provide a much more general descrtiption of the methods used to measure spermatocyte depletion only, and then provide appropriate references. This much detail is not required.  **Is the biological context (inc. taxonomic applicability/relevance, level of biological organisation) specified and explained sufficiently?**  The domain of applicability section appears to be very specific to HDIs… instead the authors should focus on what species this AO is relevent for (presumably all sperm-producing organisms)  **Has the regulatory relevance of the AO been described?**  I was unable to identify any discussion of the regulatory relevance of this AO in the document. Was it not included or is this another “snapshot” error?  **SR2:** The AO is described. It is biologically plausible, reusable for other AOPs. The biological context is provide. Number f events are described in this section: spermatocyte depletion, testicular atrophy/weight loss. Does spermatocyte depletion systematically lead to testis atrophy? Will atrophy occur after a certain level of spermatocyte depletion? Would it be worthwhile considering (extensive) spermatocyte depletion as a key event leading to testis atrophy? |

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| **Author response:**  According to the reviewers’ comments, spermatocyte depletion and testis atrophy were considered as separate key events (KE and AO). Concomitantly, the graphical representatioin was up-dated to show the key events.  The Event 1506 (AO) was renamed as “testicular toxicity” and a new KE was added to describe the spermatocyte depletion. In the new KE named “spermatocyte depletion” contains the references to have the descriptions about the effects of HDI and the AO newly renamed as testicular toxicity was revised to include the more general description and the references indicating the effects of specific chemicals on sperm production were ommitted as possible so that it can be borrowed by other AOP.  The texts in the section for how it is measured or detected were simplified in the new AO as much as possible. It was described generally how each of spermatocyte depletion and testis atrophy/weight loss in the new KE and AO, respectively, and the appropriate references were provided.  The references to specific chemicals, fixatives m reagents, etc. were ommitted as much as possible to make the AO more general and simple. The domain of applicability statement was made to be more clear and concise and focused on what species this AO is relevant for. Regulatory significane of the AO was newly addressed. |

**Section 6:**

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| **Key Event Relationships**  *Are the KERs well described and in a way that allows their use in other AOPs?*  *Are the KERs biologically plausible and is there sufficient evidence presented?*  *Is the level of empirical support adequately described in accordance with the OECD AOP Handbook?*  *Are inconsistencies, uncertainties and level of confidence adequately described?*  *Is the quantitative understanding of the KER described?"*  *[refer to Tables 2 & 3 in the handbook]* |

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| **Reviewers' responses and comments**  **PR:**  General comments: Please select taxa, sex, and life-stage applicability terms for each KER. Generally speaking, much of the right types of information has been captured, but the information could be organized in a way that would make it much easier to digest and understand. The domain of applicability section in particular is a place where the authors should aim to provide a general statement clearly stating what they think the domain of appicability is, and then support that with specific data, citations, or lines of evidence.  Relationship 1709:   * The KER description contains redundant text. This could be reduced for example by stating that HDAC inhibition leads to increased histone acetylation and gene expression and citing papers that support that. There is no need to repeat the same statement for multiple references that essentially make the same point. * Biological plausibility section lacks any supporting references. There is also reference to other post-translational modifications which do not appear to have immediate relevance to this KER or the AOP. * The empirical evidence section would be easier to read if it were presented as a set of bulleted statements rather than a long paragraph essentially listing the same. A number of the statements do not specifically address the relationship between HDAC activity and histone acetylation. For example, “MAA-induced spermatocyte death is associated with histone acetylation increase” would be better suited to support a KER (non-adjacent) linking “histone acetylation increase” to testicular toxicity. * Nice use of the uncertainties and inconsistencies section. * The information in the quantitative understanding of the linkage section is good, but clarity to the reader would likely be increased if you were to start the section with the statement “A database containing high-accuracy, species-specific phophorylation and acteylation site predictors that allows for in silico prediction of sites…..”. An important part of the relationship also involves defining how much change in HDAC activity is needed to yield some unit/percentage change in histone acetylation. To the extent possible, it would be nice if the various subsections of the quantitative understanding of the linkage could be addressed. If there is no information available (for example for known modulating factors), please just indicate “no information currently available” * The domain of applicability section contains some of the relevant information, but also some information that’s not relevant to this section. Some of the information provided here refers to the applicability of a single KE (e.g., hyperactylation or inhibtioin of HDAC), but not necessarily the connection between the two. Consider moving some of the support that pertains to just single KEs into the KE descriptions. Also, an organization of the text that starts with a clear statement of the applicable taxa, “the relationship between HDAC inhibition and hyperacetylation is likely well conserved between species from lower organisms to mammals”, and then placing bullets providing specific supporting information below.   Relationship 1710:   * The KER description should provide just a brief summation and any specialized context that might be needed. The current version includes much of what would be viewed as empirical support for the linkage, rather that a brief descriptive summation. * Empirical support section makes reference to a lot of evidence that links HDAC inhibition (exposure to HDIs) with p21 expression. Strictly speaking that information should align with a non-adjacent KER linking the MIE to p21 expression increase (relationship 1714). Empirical support for this relationship should focus on the connection between measurement of increased acetylation and increased p21 expression only. * Nice use of the uncertainties and inconsistencies section. * Relative to quantitative understanding, the authors are capturing the right kind of information, but it needs to be synthesized and presented in a more straight-forward manner for the reader. How long after histone acetylation occurs can increased p21 expression be observed? How much increase in acetylation is needed for an increase in p21 to be detected? Clear statements addressing these kinds of questions are needed, with supporting information provided to back it up. Right now, the reader gets lost in the details. The text doesn’t extract the relationship for the reader. * The domain of applicability information provided here is more relevant to relationship 1714. No reference to increased aceytlation is made.   Relationship 1711.   * The description text here is pretty good. Sticks closely to the relationship between p21 and cell cycle. * The biological plausibilty is not real clear though. Some of the KER description text could be moved into biological plausibility, with just a clear statement of what the relationship is and the context in which it operates in the KER description section. * The first sentence clearly supports the link between p21 and cell cycle disorder. The others link p21 with apoptosis and cell death without a clear explanation of how either of those relate to cell cycle. * Uncertainties and inconsistencies – it was very easy to get lost in the detail here. I wasn’t sure what the take home message was in terms of the potential uncertainties I should be aware of were. * Quantitative understanding – the informaiton contained here needs to be synthesized and extracted into a form that’s clearer and easier for a reader to use. * Domain of applicability – again, details without a sythesizing thesis statement. Provide a clear statement regarding domain, then provide detailed support as bullets, where possible.   Relationship 1712.   * In terms of biological plausibility, the right kind of information was present, but it is not organized in a way that makes it real accessible. * It was unclear how the empirical evidence cited speaks to this particular KER, same applies to the uncertainties and inconsistencies section. * The authors provide methods for measuring apoptosis and caspase activity – if anything, these should go on the event pages. They do not provide quantitative understanding of what magnitude of cell cycle disorder leads to apoptosis and under what conditions. * Domain of applicability. Support for applicability to humans and mice is provided, but a clear statement regarding the applicability of this KER is lacking.   Relationship 1713.   * The KER description should summarize the basic structural or funcational association between the two KEs being linked together. The information there right now is better suited for empirical evidence. * Biological plausibility – at present only describes what apoptosis is. It does not describe how increased apoptosis can result in testicular toxicity. * The first sentence under uncertainties and inconsistencies refers to the realtionship between HDAC inhibition and testicular toxicity, not between apoptosis and testicular toxicity. Not appropriate content for this KER page. * The information provided in the quantitative understanding does not relate a magnitude of measured apoptosis with decreased spermatocytes or reduced testis size. * Clear statement regarding the domain of applicability is lacking.   Relationship 1714.   * Consider making the KER description simpler and moving the supporting data to the empirical evidence section. * The information currently presented under biological plausibility would be more appropriate under empirical evidence (in my opinion). * The current information under empirical evidence requires more explanation. At present it is unclear how either of these sentences/statements support the KER. * The uncertainties and inconsistencies section refers to KEs that are not part of the present KER. Focus should be on the link between HDAC inhibition and p21 expresion, with possible mention of biology lying in between, but not biology that is further downstream.   Relationship 1715.   * Information provided in the KER description is better suited for empirical support. The description section should briefly define what the connection is between HDAC inhibition and cell cycle regulation. * Information in the biological plausibility section would fit better under empirical evidence.   Relationship 1716.   * The first two sentences of the KER description provide a useful summation. The rest of it should be organized under empirical evidence. * Information provided under biological plausibility is empirical evidence.   Relationship 1718.   * Information in the KER description and biological plausibility sections are probably more suited to empirical evidence. * Although the sentence under quantitative understanding provides some quantitative information, it does not address how much inhibition of HDAC is needed to yield testicular toxicity, how quickly that occurs, and whether other factors are known to modulate that relationship.   **SR1:** KER 1709: Histone deacetylase inhibition leads to Histone acetylation, increase   1. The KER is biological plausible 2. The description makes a lot of reference to gene expression… however, “gene expression” could be considered a downstream/indirect KE….    * According to KE description “Histone acetylation, increase” is measured by antibodies… this endpoint should be the focus of this KER    * Please only focus on evidence from KEup: “Histone Deacetylase Inhibition” and KEdown: “Histone acetylation, increase”. All other endpoints (ex: “gene expression”, “apoptosis”, “thioredoxin-binding protein”, etc…) are not relevant to this KER. 3. Information on the IC50 of valproic acid is too chemical specific 4. Is there any information on the Quantitative understanding of this KER?   K1710: Histone acetylation, increase leads to p21 expression, increase   1. This KER desciption is much more specifc to KEup and KEdown. This is a good example of a well-written KER description 2. It is biolgically plausible 3. Much of the emprirical evidence links increased p21 expression with HDAC inhibition…. .which is technically NOT part of this KER. KEup should be “Histone Acetylation, increase”…. I realize these are very similar.. but they are not quite the same thing.   KER 1711: p21 expression, increase leads to cell cycle disorder   1. Another excellent description that is appropriately specific to KE up and KE-down 2. The biologial plausibility section is oddly specific to hypoxia in the lungs… can it be made more general? 3. The emprical evidence makes many referrience to “apoptosis”, which is a downstream KE that is not relevent to this KER. Please only use eveidence that links p21 specifically to measured changes in cell cycle.   Relationship: 1712: cell cycle disorder leads to Apoptosis   1. Descritpion is written as “see biological plausibility”. These should not be the same thing. Please at least write a basic general description of this KER. 2. There are some “non KE-up” KEs in the description (ex: p21). 3. The emprical eveidence section is brief, but presents appropriate data.   Relationship: 1713: Apoptosis leads to testicular toxicity   1. THe KER desctription seems more like emprical evidence. Please provide a general description of the KER 2. The biological plausibility does not even mention testicualr toxicity. It describes the link between apoptosis and tumor regression…. Please make the link to testicular toxicity more clear. 3. THe empircal evidence section is very data poor and ony focuses on spermatogonial stem cells. This KER will benefit from a more specifically desribed AO   **SR2:** The KERs are nicely summarized in tables, very helpful. Then the authors have discussed the each of then, the adjacent ones and the non-adjacent ones. For the adjacent KERs, they are biologically plausible, sufficient evidence is provided with quantitative understanding and empirical support. Inconsistencies and uncertainty are discussed. For the non-adjacent ones, the “leap-over” is not obvious; especially when considering the MIE leading to the AO. Should this arrow be kept in the AOP? |

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| **Author response:**  The domain of applicability section was revised for each KER.  KER 1709: Histone deacetylase inhibition leads to histone acetylation, increase  The KER description was reduced to simply state that HDAC inhibition leads to increased histone acetylation and gene expression. The references to support biological plausibility were added in the section. The descriptions about other post-translational modifications were ommited.  The description of KER was focused on evidence from KEup. All other endpoints (e.g., “gene expression”, “apoptosis”, “thioredoxin-binding protein”) which are not relevant to this KER were omitted.  The empirical evidence section was revised to describe bulleted statements rather than a long paragraph. The sentence “MAA-induced spermatocyte death is associated with histone acetylation increase” was omitted, since it describes about KER (non-adjacent) linking histone deacetylase inhibition to testicular toxicity.  The quantitative understanding of the linkage section was started with the statement “A database containing high-accuracy, species-specific phosphorylation and acetylation site predictors that allows for in silico prediction of sites…….”. The descriptions were revised with bulleted statements.  The domain of applicability section was revised, where the description about the applicability of a single KE (e.g., hyperacetylation or inhibition of HDAC) were moved to the descriptions of KE. For the applicable texa, the text was started with a clear statement describing “the relationship between HDAC inhibition and hyperacetylation is likely well conserved between species from lower organisms to mammals”, and then bullets providing specific supporting information were placed.  The information on the IC50 of valproic acid derivatives was revised to be more general.  KER 1710: Histone acetylation, increase leads to p21 (CDKN1A) expression, increase  The KER description was revised to provide a brief summation and specialized context and not include too much information. The empirical support section was revised to focus on the connection between measurement of increased acetylation and increased p21 expression only.  The quantitative understanding was revised to be more straight-forward manner. The time span of p21 expression increase after histone acetylation and the extent of the acetylation increase needed for an increase in p21 were added with supporting information.  The domain of applicability was revised to cite references for increased histone acetylation leading to p21 expression increase. The description about p21 expression increase by HDAC inhibition was moved to KER1714.  KER 1711: p21 (CDKN1A) expression, increase leads to cell cycle, disrupted  The biological plausibility was revised to be more clear and general not to be specific to hyperoxia in the lungs. The part of the KER description text was moved into biological plausibility, with clear statement of what the relationship is and the context in which it operates in the KER description section.  The sentences other than the first sentence were revised to link p21 and cell cycle, not apoptosis.  Uncertainties and inconsistencies were revised to clarify the potential uncertainties.  Quantitative understanding was revised to be synthesized and extracted into a form that’s clearer and easier for a reader to use.  Domain of applicability was revised to provide a clear statement regarding domain and detailed support as bullets.  The empirical evidence was revised to be more relevant to p21 and cell cycle linkage, not apoptosis linkage.  KER 1712: cell cycle, disrupted leads to apoptosis  The biological plausibility was revised to be organized in a way that makes it real accessible. The description about the p21 was deleted, and the description about the desregulation of cell cycle inducing the apoptosis was moved to KER description.  The empirical evidence was revised to add the linkage between the cited reference and KER.  The uncertainties and inconsistencies was revised to clearly note the KER.  The methods for measuring apoptosis and caspase activity was moved to the event pages.  The quantitative understanding of what magnitude of and under what conditions cell cycle disorder leads to apoptosis was added.  Domain of applicability was revised to clearly state the applicability of this KER.  KER description was revised to explatin the KER as follows:  “Cell cycle dysregulation leads to apoptosis. microRNA-497, potentially targeting Bcl2 and Cyclin D2 (CCND2), induced apoptosis via the Bcl-2/Bax - caspase 9 - caspase 3 pathway and CCND2 protein in human umbilical vein endothelial cells (HUVECs) (Wu R 2016). The microRNA-497 activated caspases 9 and 3, and decreased Bcl2 and CCND2 (Wu R 2016). CCND2 is an important cell cycle gene that induces G1 arrest (Li L 2012), and deregulated CCND2 is implicated in cell proliferation inhibition (Wu R 2016, Mermelstein A 2005, Dong Q 2010).”  KER 1713: apoptosis leads to testicular toxicity  This KER was revised to create two KERs, which are KER 1735: apoptosis leads to spermatocyte depletion, and KER 1734: spermatocyte depletion leads to testicular toxicity, since new KE1515: spermatocyte depletion was created.  KER description was revised to summarize the basic structural or functional association between the two KEs and provide the general description of the KER. The part of the description in KER was moved to the empirical evidence.  Biological plausibility was revised to include how increased apoptosis can result in spermatocyte depletion.  The empirical evidence was revised to describe testicular toxicity with additional data.  The first sentence under uncertainties and inconsistencies was revised to refer to the relationship between apoptosis and spermatocyte depletion, not between HDAC inhibition and testicular toxicity.  The quantitative understanding was revised to provide the information relating a magnitude of measured apoptosis with decreased spermatocytes.  The domain of applicability was revised to clearly state the domain.  KER 1714: Histone deacetylase inhibition leads to p21 (CDKN1A) expression, increase  KER description was revised to make simpler and the supporting data was moved to the empirical evidence.  The part of the information in biological plausibility was moved to the empirical evidence.  The empirical evidence was revised to add more information supporting the KER, and the part of the information in KER description and biological plausiblity were moved into the empirical evidence.  The uncertainties and inconsistencies was revised to refer to KEs in the present KER, not the KEs not relating the KER. The description was focused on the link between HDAC inhibition and p21 expression with biology information between them not in the further downstream.  KER 1715: Histone deacetylase inhibition leads to cell cycle, disrupted  The part of the KER description was moved to the empirical support. The description was revised to define the connection between HDAC inhibition and cell cycle regulation.  The part of the information in the biological plausibility was moved to the empirical evidence.  KER 1716: Histone deacetylase inhibition leads to apoptosis  The descriptions except for the first two sentences of the KER description were moved to the empirical evidence.  The part of the information in biological plausibility was moved to empirical evidence.  KER 1717: Histone deacetylase inhibition leads to testicular toxicity  Information in the KER description and biological plausibility were moved to empirical evidence.  The quantitative understanding was revised to add the information addressing the extent of the inhibition of HDAC needed to yield testicular toxicity, and how quickly that occurs, and whether other factors are known to modulate that relationship.  [Overall]  The arrow from KE1 towards apoptosis was revised to begin with MIE in the graphical representation. The arrow from MIE to AO was deleted. The new KER titled spermatocyte depletion leads to testicular toxicity was added, according to the reviewers’ comments to create a new KE titled spermatocyte depletion. |

**Section 7:**

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| **Overall Assessment of the AOP**  *Is the domain of applicability of the AOP defined appropriately?*  *Is the level of support for essentiality of the KEs adequately described and assessed?*  *Has the degree of quantitative understanding of KERs been assessed properly?*  *Has consideration been given to the overall weight of evidence for the AOP?*  *Are the calls on Overall WoE and Quantitative Understanding supported?* |

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| **Reviewers' responses and comments**  **PR:**   * Life stage and sex applicability are defined in the structured fields on the AOP page, even though that information is missing on the KE and KER pages. * The level of support for essetiality of the KEs and the overall rationale for the evidence calls provided in the KER table is not very well described here. I would urge the authors to take a closer look at Section 4 of the handbook, and the guiding questions provided in Annex 1 and Annex 2. It is not necessary to repeat supporting data here. What is most important is to provide the rationale for assigning calls of high, moderate, or low. * There is no need to copy the structured domain of applicability tables to the free-text domain of applicability section. This section should be used to provide any additional rationale or characterization that is not readily expressed in the machine-readable structured fields. * The overall assessment of the AOP needs some work.   **SR1:** THe weight of evidence section is complete void of any text that justifies the WOE calls that the authors have made. This information is essential.  Further, the empirical evidence section on the individual KER should be improved. The authors should more clearly indicate which type of evidence is being presented. It is recommended that each “empricial evidence” section for each KER have subsections titled “dose-concordance”, “temporal concordance” and “incidence concordance”.  Please see page 41 of the Handbook (2017 version)  **SR2:** The overal assessment of the AOP is clear. The tables provide in this section are very helpful, thank you. |

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| **Author response:**  Thank you very much for the comments. The information about the life stage and sex applicability was added to the KE and KER pages.  The description about the level of support for essentiality of the KEs and the overall rationale for the evidence calls provided in the KER table were added. Section 4 of the handbook and the guiding questions in Annex 1 and Annex 2 was re-checked, and the repeated supporting data were deleted, and the rationale for assigning calls of high, moderate, or low were added.  The structured domain of applicability tables in the free-text domain of applicability sections was deleted. The section was revised to provide the additional rationale or characterization that is not readily expressed in the machine-readable structured fields.  The overall assessment of the AOP was revised.  The weight of evidence section was revised to include the information justifies the WOE calls .  The empirical evidence section on the individual KER was reviesed to clearly indicate which type of evidence is being presented. The each “emperical evidence” section for each KER was revised to have subsections titled “dose-concordance”, “temporal concordance” and “incidence concordance”. The page 41 of the Handbook was re-checked. |

**Section 8:**

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| **Potential application of the AOP (optional):**  *Is any context provided as regards the reason for development or the intended use?* |

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| **Reviewers' responses and comments**  **PR:** Yes, some context and rationale is provided.  **SR1:** A very geneeral statement is provided. Are there any specifc examples or relevant test guidelines?  **SR2:** The authors suggest an application of the AOP for investigating the risk assessment of HDIs for testicular toxicity. |

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| **Author response:**  The specific example was added. |

**General Observations and Conclusions of the Reviewer**

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| **Reviewers' responses and comments**  **PR:**  Generally speaking the AOP seems plausible and lots of good supporting information has been collected. However, at present much of it is just presented as long lists of facts/supporting information. This is particularly true on the KER pages. The facts provided are often not translated in a way that makes the key points clear to a reader who is unfamiliar with all the details. It is recommended that the authors should focus on re-organizing many of the sections into a format in which a clear statement about the domain of applicability, different types of support, etc. are made for a KER, then the supporting data/details are provided as bullets below. As suggested by SR2, organizing the empirical evidence into subsections for data that address dose-response concordance, temporal concordance, etc. specifically may help. Overall, the goal is not to simply throw up as much data as possible and then let the reader sort it out, but rather to communicat the story as simply as possible, while also providing the data and references that back up the clear concise statements. In its present form, much of this AOP will be inaccessible to many readers, even those with a reasonable background in biology. That will make the AOP difficult to use. Consequently, time spent making the key points easier to pick out and understand will have significant benefits in the longer term.  With regard to key events, the probable taxonomic domains of applicability should probably be broadened. Even though data supporting this AOP may have been primarily derived from human, mouse, and rat models, these are pathways that are likely conserved across a broad range of eukaryotic organisms. Early key events are probably even relevant to a number of species that lack testes. While a narrow range of taxonomic applicability may be defined using structured fields, it would be useful to try to define the probable taxonomic domain and a rationale for it in the free text section of the pages.  SR2 raises a good point that there are numerous existing KEs for apoptosis in the AOP-Wiki. It might be worth taking a look at some of those other pages and see if there is an existing one, already connected to other AOPs in the wiki that would be suitable for the current AOP. If so, such consolidation can aid in network building etc.  The authors have done a nice job of filling in the KER tables with “calls” of High/Moderate/or Low for Evidence and Quantitative Understanding. However, it is important that the rationale/justification for these calls be presented on the AOP page (overall assessment of the AOP section). It is not necessary to reiterate all the data assembled on the KER pages, etc. However, the authors should consider the guiding questions laid out in Annex 1 and Annex 2 of the “User’s Handbook” ( <https://one.oecd.org/document/ENV/JM/MONO(2016)12/en/pdf)>, and explain how consideration of those questions led to selection of high/moderate/or low weight of evidence.  Finally, although this was perhaps not described in the Handbook or available in the AOP-Wiki when this AOP description was first developed, it would be useful for the authors to select “Key Event Component” terms relevant to their KEs. This aids in making the AOPs more machine-readable for the purpose of AOP network construction.  **Overall, I would recommend that the AOP (and associated KE and KER) description be revised before moving to the external review stage.** However, based on what the authors have assembled, with some reorganization and a bit more synthesis and explanation of the current content, I would envision that this AOP may be able to move to external review after the major comments outlined here and by SR1 and SR2 have been addressed.  Thank you for your patience. My apologies for the short delay in providing my review.  **SR1:** Overall I think this is a very usefull AOP. There is a very clear regulatory need for this AOP. It also provides very many KE and KERs that can be borrowed by future AOP authors. However, there are several shortcoming that need to be addressed. Some specific examples are:   1. Some of the KEs and KER descriptions are very specific to HDIs… making them a little less easy to borrow. I suggest making these description more general 2. THe AO is not very clearly defined…. is it only releevnt to spermatocytes? Furthermore, I suggest that the AOP be extened to include at least one additional, more general AO with more clear regulatory applciation (ex: reduced sperm count, or reduced fertility) 3. The empitical evidence sections in each KER need to be organized so the the type of evidence being present is more clear. It is recommended that each “empirical evidence” section of each KER have the following subsections: “dose-concordance”, “temporal concordance” and “incidence concordance”, and that each of these sections only contain data relevent to the two KEs involved in the KER. See Page 41 of the Handbook (2017 version) 4. Finally, the WOE summary of the AO is completely void of an explanation for the WOE scores. This information must be provided.   **SR2:** The rationale for developing this AOP is very robust. The authors have systematically documented the MIE, KEs and the AO, as well as the KERs.The document may gain in clarity by correcting a few typos and adding an abbreviation section at the beginning of the description of the AOP. A few (minor) issues may be addressed to get it ready for the external review. |

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| **Author response:**  For PR: Thank you very much for valuable comments for this AOP. According to the reviewers’ comments, the KERs were updated to focus on re-organizing many of the sections into a fformat in which a clear statement about thte domain of applicability, different types of support, etc. are made for a KER with bullets indicating supporting data or details. The rationale for the probable taxonomic domain was described in the free text section of the pages.  The rationale or justification for the calls of evidences were added to strength the AOP. The Annex1 and Annex 2 of the “User’s Handbook” were re-checked and reflected in the text.  The AOP and associated KE and KER description were revised to indicate the linkages of the KEs and the evidences of the KERs.  For SR1: Thank you very much for your comments. The KEs and KER descriptions which are specific to HDIs were revised to be more general. The AO was revised to be testicular toxicity, and a new KE was added as spermatocyte depletion with more clear regulatory application. The empirical evidence section in each KER was revised to be organized so that the type of the evidences is clearer. Each “empirical evidence” section of each KER was revised to include the sections “dose-concordance”, “temporal concordance” and “incidene concordance”, and the data relevant to the two KEs involved in the KER. WOE summary for the AO was revised to include the explanation for the WOE scores.  For SR2: Thank you very much for your comments. The document was revised to corrrect typos and add an abbreviation section at the beginning of the description of the AOP. |