Sharples, A., Turner, D. (2023). Skeletal Muscle Memory. *American Journal of Physiology - Cell Physiology, 324*(6), c1274-c1294. http://dx.doi.org/10.1152/ajpcell.00099.2023

Skeletal Muscle Memory

Adam P. Sharples[1] and Daniel C. Turner[1]

[1] Institute for Physical Performance, Norwegian School of Sport Sciences, Olso, Norway.

Correspondence:

Adam P. Sharples: a.p.sharples@googlemail.com

Daniel C. Turner: danieltu@nih.no

Abstract

Skeletal muscle memory is an exciting phenomenon gaining significant traction across several scientific communities, amongst exercise practitioners and the public. Research has demonstrated that skeletal muscle tissue can be 'primed' by earlier positive encounters with exercise training that can enhance adaptation to later training, even following significant periods of exercise cessation or detraining. This review will describe and discuss the most recent research investigating the underlying mechanisms of skeletal muscle memory: 1) 'cellular' muscle memory and, 2) 'epigenetic' muscle memory, as well as emerging evidence of how these theories may work in synergy. We will discuss both 'positive' and 'negative' muscle memory and highlight the importance of investigating muscle memory for optimising exercise interventions and training programmes as well as the development of therapeutic strategies for counteracting muscle wasting conditions and age-related muscle loss. Finally, important directions emerging in the field will be highlighted to advance the next generation of studies in skeletal muscle memory research into the future.

New & Noteworthy

In this article we provide the most comprehensive review of the advances in skeletal muscle memory research to date.

What is muscle memory? An introduction

Whenever we hear the term ‘memory’, we think about the recollection of a specific event encountered in our lives that is stored in the brain. Within wider biology, another familiar example of ‘memory’ would apply to our immune system, that retains the ability to produce antibodies to protect against specific antigens it has been exposed to in the past. When specifically referring to ‘muscle memory’, we perhaps associate this with the ability to reperform certain movements or motor skills, such as riding a bike. A skill that is learnt and typically not forgotten. However, the concept of muscle memory has now evolved amongst scientists and across the wider public domain. This evolution extends the concept of muscle memory and relates to a type of memory that resides at the cellular and molecular level in the skeletal muscle tissue itself. This is therefore perhaps a more accurate description of the term muscle memory as opposed to the acquisition and retention of a learnt motor skill that resides primarily in the central nervous system and is therefore associated with a motor learning or memory. Despite this, the knowledge of motor learning preceded an understanding of muscle memory that resides in skeletal muscle tissue and therefore the synonymous use of the term across both contexts is understandable. This review will focus on muscle memory that resides at the cellular and molecular level in the skeletal muscle tissue itself.

The relevance of understanding skeletal muscle memory is that this specific organ is the most abundant, malleable, and adaptable adult tissue that responds sensitively to various environmental stimuli (**Figure 1**). Specifically, engaging in physical activity and exercise positively improves muscle size, metabolic processes, and functional performance. Equally, muscle tissue mass and strength is rapidly compromised in response to periods of physical inactivity (e.g. injury, disuse, hospitalisation), and various disease states (e.g. cancer and diabetes) alongside a gradual and natural deterioration with age, a disorder called sarcopenia (reviewed in (1)). There have been various observations that encounters with positive and negative environmental stimuli may be ‘remembered’ by the cells that make up muscle tissue, and that our muscle can respond differently if these stimuli are encountered again later. Therefore, in this context, skeletal muscle memory has been defined as: T*he capacity of skeletal muscle to respond differently to environmental stimuli in an adaptive (positive) or maladaptive (negative) manner if the stimuli have been encountered previously*’ (2). In the context of exercise, muscle responds and adapts in an advantageous manner, where the associated molecular and phenotypic changes to exercise training are accentuated when a similar exercise stimulus has been performed previously. Specifically, the muscle hypertrophic and functional response to resistance training occur more quickly and to a greater extent following a second period of retraining, even when undertaken after a prolonged period of physical inactivity or ‘detraining’ where muscle mass has returned to pre-training levels (3, 4). This suggests that earlier training is able to ´prime´ skeletal muscle for a greater response and adaptation to later retraining, even following several months of detraining, a concept that was identified in the early 1990s (3). While some of the greater adaptative responses in strength to later retraining can be, in part, attributed to improved neural activation of the muscle, advanced molecular studies conducted over the past decade have demonstrated that the

cells within our muscle may also possess a memory of earlier training-induced muscle growth. Currently, there are two main underlying mechanisms proposed to be responsible for muscle possessing a memory of 'positive' stimuli: 1) The 'cellular' mechanism of muscle memory that relates to the accrual of new nuclei within muscle fibres (myonuclei) following a period of muscle growth that are maybe retained even during subsequent periods of muscle loss and are then associated with enhanced adaptation to later re-growth (5) (**Figure 1**) and: 2) The 'epigenetic' mechanism of muscle memory which relates to modifications to skeletal muscle DNA following earlier exercise-induced muscle growth, modifications of which are retained even into detraining where exercise is ceased, and is associated with enhancing the molecular responses and thus adaptation to later retraining (4, 6, 7) (**Figure 2**).

It is also plausible to hypothesise that a 'negative' muscle memory may also exist, whereby exposure to a muscle wasting-stimulus such as disuse or immobilisation due to injury or hospitalisation may result in the muscle becoming more susceptible to further muscle wasting if the same (or similar) stimuli is reencountered. *In vitro* experiments provide proof-of-concept for a negative muscle memory, where cultured muscle cells retained epigenetic alterations after treatment with high dose inflammatory cytokine, Tumour Necrosis Factor Alpha/TNF-α, at levels mimicking muscle wasting conditions such as cancer cachexia. Consequently, when retreating with TNF-α at a late stage in the muscle cells proliferative lifespan, differentiation capacity and myotube growth was further compromised (8). Evidence from animal studies also demonstrates that *in-utero* malnutrition leads to epigenetic changes and associated alterations in muscle size, fibre type and function of the offspring, suggestive that growth restricting environments during development and even before birth can negatively impact skeletal muscle in later life (reviewed in (2, 9)). Finally, to the authors knowledge, there is only one study that (indirectly) points towards the notion of negative muscle memory in humans, whereby breast cancer patients retain epigenetic modifications to skeletal muscle DNA (via DNA methylation) over 10 years after diagnosis and treatment (10). However, so far there is very little direct evidence in adult humans of a negative memory in skeletal muscle tissue.

The purpose of this review is to describe the current working theories of cellular and epigenetic muscle memory in isolation as well as the emerging evidence of how these theories may also work in synergy. Beyond the direct implications for muscle memory research, to help elucidate the underlying mechanisms of skeletal muscle adaptation, we will also outline the practical implications of muscle memory for optimising exercise interventions and training programmes for athletes and the general population as well as the development of therapeutic strategies for counteracting muscle wasting conditions and age-related muscle loss. Finally, important directions emerging in the field of skeletal muscle memory and the importance of investigating 'negative' muscle memory for muscle wasting conditions will be highlighted to advance the next generation of studies in this field.

Theories for the mechanistic understanding of muscle memory

Cellular memory: An introduction

In 2013, work by Professor Kristian Gunderson's laboratory first provided evidence that skeletal muscle could 'remember' hypertrophic stimuli. This study treated mice with testosterone for 14 days which led to an increase in muscle size and myonuclei number within the muscle fibres (5). Then, after a 3-month testosterone 'washout' period and testosterone abstinence (equivalent to ~12% of the mice's lifespan), muscle size returned to pre-testosterone levels. Following the 3 months of testosterone abstinence, mechanical overload was performed using a procedure called synergistic ablation or mechanical overload, which involves the surgical removal of synergistic muscles leading to supraphysiological loading and subsequent rapid hypertrophy of the remaining intact muscles of the same muscle group (11). Interestingly, mice that had received earlier testosterone demonstrated a 31% increase in extensor digitorum longus (EDL) muscle cross-sectional area (CSA) after 6 days mechanical overload compared with a non-significant 6% increase in growth observed in control mice that did not receive earlier testosterone (5). The EDL muscle between the two groups grew similarly for the remaining 8 days of overload, although the overall growth after 14 days of overload was still 20% greater in the testosterone versus non-testosterone treated mice (5). Collectively, these findings suggested that earlier testosterone treatment allowed skeletal muscle to grow more quickly and to a greater extent following later mechanical overload, suggestive of a positive muscle memory from earlier testosterone use (**Figure 1**). The authors attributed the enhanced adaptation to mechanical overload to the retention of myonuclei from the earlier testosterone administration. However, this direct conclusion has been somewhat controversial because even though testosterone treatment did increase the number of myonuclei compared with sham controls, there was a reduction in the number of nuclei per fibre in the EDL after the testosterone washout period in testosterone treated mice. Despite this, the study still satisfies the current working definition of muscle memory described above, where an earlier exposure to a positive environmental stimulus evoking hypertrophy (anabolic stimulus of testosterone) led to an enhanced adaptive response when another hypertrophic stimulus (mechanical overload) was reencountered in the future.

Why are increased myonuclei important for muscle adaptation?

Before discussing more evidence of cellular muscle memory in detail, it is important to clarify why myonuclei incorporation into muscle fibres maybe important for the enhanced adaptive response to later environmental stimuli. Skeletal muscle contains fascicles that each encompass up to hundreds of muscle fibres, and fibres can be up to 20 cm in length and 10 mm^2 in area (for example, in the human sartorius muscle in the thigh) (12). It is therefore estimated that 1 long fibre could contain tens of thousands of myonuclei and muscle fibres are therefore classed as highly multinucleated cells. This is crucial, as nuclei within our cells contain our DNA and are therefore an important site for where our

muscle fibres get their 'blueprint' to transcribe genes in the nucleus. The molecular information from our DNA is then transcribed and translocates out of the nucleus as messenger RNA (mRNA) to synthesize corresponding proteins in the ribosome. The proteins synthesized in the ribosome are subsequently important to enable adaptation of muscle tissue such as an increase in size and function, for example, via increases in the abundance of contractile and structural proteins. Therefore, 'myo'-nuclei simply refers to nuclei that reside within muscle fibres. As we have described, muscle fibres contain many myonuclei within each fibre, however, under normal physiological conditions, adult muscle fibres cannot undergo division to create a brand-new fibre (a process termed hyperplasia) and muscle fibres are therefore deemed to be 'terminally differentiated'. That is, muscle fibres cannot undergo mitosis that is possible in cells containing a single nucleus. Given muscle fibres adapt positively to exercise, this begs the question, how is the adaptation of skeletal muscle achieved without being able to add new fibres? This is in part due to a resident muscle-specific adult stem cell, initially identified by Alexander Mauro and Bernard Katz in frog muscle (13, 14). Since their initial discoveries, these cells are often referred to as ´satellite´ cells, the term coined by Mauro, owing to their peripheral 'satellite' location between the sarcolemma and basal lamina of muscle fibres. It was later demonstrated that upon activation, these single cells can undergo cell division, particularly following a stimulus or insult such as injury or muscle damage from heavy resistance exercise, where they then migrate to the site of damage and fuse to the existing muscle fibre and contribute new myonuclei (15-23). Indeed, satellite cells are the main source of new myonuclei in muscle after exercise (24-26). Very recent data has also suggested that a small percentage (1-3%) of myonuclei can undergo a type of endoreplication within the muscle fibres (27), also perhaps contributing to an increase in myonuclei number with mechanical loading. Therefore, it would still be a general assumption that the majority of new myonuclei are incorporated into the muscle fibre via satellite cell fusion, and satellite cells are therefore considered crucial for increases in muscle size after hypertrophic stimuli. However, this assumption has proven controversial.

Are new myonuclei from satellite cells required for hypertrophy?

Within the field of muscle cell biology, there is controversy as to whether satellite cells are required for load or exercise-induced muscle growth. This is because some animal studies have demonstrated that satellite cells are not always required for muscle growth after exercise mimicking stimuli whereas others suggest satellite cells are important contributors to hypertrophy. Early studies attempting to address this used gamma irradiation to ablate satellite cells and provided evidence that without satellite cells there was impaired load-induced hypertrophy of skeletal muscle (28). However, the specificity of gamma irradiation on satellite cells *per se* led to the development of pax7 specific knockout animals, a genetic model that could specifically ablate satellite cells in muscle tissue. Indeed, John McCarthy and Charlotte Peterson's groups reported a normal hypertrophic response after mechanical overload via synergist ablation in rodents after using this model of genetic ablation of satellite cells, suggesting that newly acquired myonuclei from satellite cells may not be essential for load-induced hypertrophy of skeletal muscle (29), at least when hypertrophy is assessed over shorter

compared with longer periods, discussed below. However, groups attempting to repeat these experiments have demonstrated that hypertrophy was prevented when satellite cells were depleted (30). These studies have led to debates in the literature regarding methodological approaches of the models used, that still extends to recent times (31, 32).

Despite this controversy, it is also now understood that myonuclei already fused into fibres can also increase their transcriptional output to serve an increase in 'myonuclear domain' size (33, 34). Myonuclear domain size refers to the area of cytoplasm each myonuclei serves within the muscle fibre. Therefore, hypertrophy occurring via expansion of cytoplasm around each myonucleus via an increase in its transcriptional (mRNA and/or rRNA (35)) and thus translational output, before the muscle fibres require fusion of new nuclei from satellite cells, may enable the growth of muscle without the requirement and addition of new myonuclei. This process has been described as the existing myonuclei possessing a 'reserve' capacity (34). Despite this, it is also the case that muscle with 50% fewer nuclei, manipulated experimentally via the use of myomaker knockout rodents that are fusion incompetent, have smaller muscles than wild-type controls (2). Also, if nuclei are depleted by 75% then muscle can become non-functional and even pathological (2). This therefore suggests that muscle fibre size during development is limited by the nuclear DNA content that is present in myonuclei and therefore the number of myonuclei in fibres is closely and positively associated with muscle size (33, 34). It is also worth pointing out that paradigm shifting data is emerging that suggests myonuclei within fibres may not be post-mitotic and may actually replicate at low rates of 1% under normal conditions and up to 3% after synergistic ablation, where this replication may occur via endoreplication and polyploidy (27). This process perhaps thought to occur instead of normal mitosis via nuclear division and cytokinesis, where endoreplication would be required to maintain the highly organized contractile elements of skeletal muscle tissue whilst facilitating an increase in myonuclei number (27).

Whether myonuclear replication occurs in human skeletal muscle after physiological stimuli of exercise remains unknown. Despite this, it seems that the number of satellite cells and the proportion of myonuclei number to cytoplasmic volume are important determinants of muscle size and as well as maintenance of its structure and function. Indeed, the proportion of myonuclei to cytoplasm have been proposed to be a key factor in muscle fibre size increases rather than 'more is better', as discussed in a recent insightful review on the myonuclear domain hypothesis (36). It is also typical that animal studies investigate mechanical overload-induced hypertrophy over a period of days and it appears that if satellite cells are removed for longer periods, such as 8 weeks, then the replacement of myonuclei is likely to be impaired and the muscle can become fibrotic (37). This is presumably due to a lack of satellite cells undergoing myogenesis contributing to the production of contractile proteins and instead infiltration and production of excessive extracellular matrix (ECM) proteins by fibroblasts. In support of these studies, other groups also suggest that the inability of satellite cells to fuse (in myomaker knockout mice) leads to fibrosis after synergistic ablation-induced hypertrophy (38). The same occurs when satellite cells cannot fuse following high intensity exercise (that usually causes

hypertrophy) and results in impaired muscle function and exercise intolerance (39). Further, satellite cells, even in the absence of fusion, have been demonstrated to communicate to interstitial fibrogenic cells and the muscle fibres themselves to co-ordinate proper ECM deposition and muscle fibre remodeling in response to hypertrophic stimuli. This may further suggest an important role for satellite cells in muscle ECM and fibre remodeling even when they do not fuse to muscle fibres (40, 41). Indeed, in pax7 knockout rodents that deplete satellite cells, even though some hypertrophy does occur following 4-8 weeks progressive weighted wheel running (PoWeR) in rodents, hypertrophy is blunted compared with conditions where satellite cells are present (35). Further, transcriptional profiling also demonstrates several gene networks that are altered in the absence of satellite cells, and ultimately growth and adaptation are blunted after 4-8 weeks of exercise (35). Overall, these studies suggest that satellite cells are probably required to maintain any longer-term increases in muscle size and function following load induced growth or exercise. Finally, it is also worth noting that most of the studies in this area have been in rodents, where human muscle fibres, on average, are larger (mean fibre area approximately 4,500 μm^2) compared with mouse muscles (mean fibre area of approximately <1,500 μm^2). Studies in humans have suggested that growth of larger fibres compared with smaller fibres seems to be more dependent on satellite cells as the muscle increases in size, whereas hypertrophy of smaller fibres may be more associated with changes in myonuclear domain size (42, 43). Indeed, it has been demonstrated that the increase in size of small muscle fibres (2,000–4,000 μm^2) in humans after 12 weeks of resistance training was correlated with an increase in myonuclear domain, whereas an increase in the size of the largest muscle fibres (8,000–10,000 μm^2) correlated with an increase in myonuclear number (42, 43). This therefore supports the notion discussed above regarding a 'reserve' capacity, where existing myonuclei increase their transcriptional and translational output up to a certain myonuclear domain ceiling size, meaning hypertrophy can occur in small fibres without the need for accretion of myonuclei from satellite cell fusion, whereas growth in larger fibres following resistance exercise in humans may require accrual of new myonuclei.

How is the controversy over the importance of satellite cells in hypertrophy relevant to muscle cell memory?

It is important to discuss the controversy outlined above, as the understanding of the limitations within the satellite cell biology field extends to studies investigating muscle cell memory. This is because, if satellite cells (or even a small contribution of new myonuclei from endoreplication (27)) and therefore myonuclear accretion were not required for hypertrophy then there can essentially be no retention of myonuclei to form a cellular memory of earlier hypertrophy. However logically, and supported by experiments, when satellite cells are present (and not depleted) then myonuclear accretion tends to occur. Indeed, some studies using synergistic ablation in rodents have demonstrated that increases in the number of myonuclei occurring in the mouse EDL muscle are retained (i.e., myonuclei number remains elevated) during a subsequent period of muscle atrophy caused by denervation (44), and that progressive weighted wheel running (45) and progessive loaded ladder climbing in rodents (46, 47)

also evokes and increase in muscle size and myonuclear accretion. With recent meta-analysis showing that, on average, across experimental models of hypertrophy in rodents, myonuclear accretion increased by approximately 23% after the initial loading stimuli and that myonuclear accretion is increased on average about 9% after training in humans (48). Therefore, it seems clear that myonuclear accretion occurs in most animal models of hypertrophy (syngergist ablation, ladder climbing, weighted wheel running) and in humans after resistance training at least from these recent meta-analyses. Perhaps the controversy that lies in the muscle memory field is whether newly accrued myonuclei are retained or lost after a subsequent period of muscle loss following the earlier growth period. As discussed above, studies have suggested that newly accrued myonuclei after testosterone treatment maybe retained during subsequent testosterone abstinence (5), and increased myonuclei following mechanical overload in mice were also retained during a subsequent period of denervation-induced muscle atrophy (44). It therefore seems that experiments using testosterone and synergist ablation to evoke hypertrophy may lead to the retention of myonuclei that are not lost during periods of atrophy. However, the main limitation of these models is whether the supraphysiological hormonal stimuli of testosterone or synergistic ablation in rodents translates to the physiological response to exercise in human skeletal muscle. Therefore, studies using more physiologically relevant models of exercise induced hypertrophy such as PoWeR in mice have demonstrated that the myonuclei accreted from weighted wheel running were not retained after a period of subsequent detraining (45). This model of weighted wheel running evokes a hypertrophic response in predominantly oxidative fibre types. Therefore, this type of exercise may not be entirely representative of hypertrophy that occurs in faster fibre types after higher load resistance exercise. To address this, a recent study utilised a physiologically relevant resistance exercise protocol in rodents to assess whether any enhancements in muscle mass were associated with an increase in the number of myonuclei after the initial training period, and if these were retained or lost following a subsequent period of detraining (46). In this study, rats underwent 8 weeks of progressive loaded ladder climbing. This included 3 sets of 5 reps starting at 50% of body weight that gradually increased to 300% of body weight, twice per day, every third day for 8 weeks (46). This training period was then followed by 20 weeks of detraining and finally another 8 weeks of retraining (46). As with the previous studies, myonuclei were accrued with training and importantly the authors demonstrated that the number of myonuclei were retained even throughout detraining, supporting the cellular muscle memory hypothesis in a physiologically relevant model of hypertrophy in rodents. In this study however, there were no larger increases detected in all measures of muscle size after later retraining (i.e., CSA and absolute mass). However, the authors did report that relative muscle mass was enhanced after later retraining (46). In further support of these data, a study utilised a 5 week ladder climbing protocol in rats, followed by 10 weeks of detraining and then a further 2 weeks retraining (using either ladder climbing or mechanical overload/synergist ablation) resulting in increased muscle size and myonuclei number after training that were retained during detraining even when muscle mass returned to pre-training levels (47). Interestingly, although the increase in muscle size after the later retraining did not reach the same levels as the earlier training period, perhaps due to the shorter retraining period compared to initial training, there was an even greater increase in myonuclei number after retraining

(47). The authors therefore concluded that the earlier training produced a retained myonuclear number during detraining that was 'boosted' in response to later retraining. A caveat with both of the above rat studies is that the first training periods are undertaken in juvenile rodents, and therefore whilst this provides evidence of mynonuclear retention following early life exercise, it would be important to perhaps confirm this in adult rodent muscle using the same physiologically relevant models of hypertrophy. To summarise, many of the animal models that use more physiologically relevant exercise training regimes of higher load resistance training have demonstrated cellular memory by myonuclear accretion and retention in muscle tissue.

Is there a cellular muscle memory in humans after training?

Given the rodents studies discussed above, the next logical question is whether the same retention of myonuclei occurs in human muscle following a period of training and detraining, and whether this is associated with enhanced adaptation to later retraining. The first work in humans to begin to address this question demonstrated that 3 months of resistance training (3 times/week) increased muscle fibre size and satellite cell number (49), where the increase in satellite cell number remained elevated for 10 days even after exercise cessation and detraining. However, by 90 days post-detraining, satellite cell number had returned to baseline levels (49). Therefore, suggesting that there was a retention of the increased number of satellite cells after training, however, that this was not the case for longer periods of 90 days. It is important to highlight that the authors did not report the number of myonuclei in this study, where some of the increased number of satellite cells may have subsequently be incorporated into the muscle fibres to serve as myonuclei. The most relevant and recent study to address whether a cellular memory of hypertrophy exists in human skeletal muscle was a collaborative venture by Niklas Psilander, Kristian Gundersen and Truls Rastaad's laboratories (50). In this study, myonuclei number was assessed in the quadriceps muscle before and after 10 weeks of resistance training. The training consisted of 3 times per week unilateral leg extension and leg press exercise at 70–85% 1RM with the non-exercise leg serving as a contralateral control limb. After the initial training period, participant underwent 20 weeks of detraining, followed by 5 weeks of bilateral retraining (50). Myonuclei number and fibre volume were not altered after the first training period, despite an increase in overall muscle size and strength, suggesting that the initial training period did not adequately induce the expected adaptation to assess properly if there was a cellular memory from the earlier training into the later detraining and retraining periods (50). These findings, together with the accessibility of the raw data, meant that others were able to reanalyse the data set and publish their own interpretations of this study as a viewpoint article in the *Journal of Applied Physiology* (51). The debates in the results gained further traction across the scientific community where several expert muscle scientists published their comments in the same issue (52). However, due to the findings in the original study and the various controversies, it was therefore not possible to decisively conclude whether a cellular memory via retention of myonuclei occurs in human muscle after resistance training.

Other recent studies with similar designs have also been conducted in elderly humans following 12 weeks of progressive resistance training followed by the same period of detraining and retraining (53). The authors investigated myonuclei alterations to identify if there was evidence of a cellular muscle memory in this older population (53). In this study, resistance training did not significantly increase myonuclear number in either type I or type II fibres. Albeit on an individual level, a small number of individuals increased myonuclei number, while others demonstrated no change, and some even demonstrated a reduction in myonuclei. However, the study did demonstrate the detraining period evoked a significant decrease in myonuclear number in both type I and type II fibres. Something that had been observed previously in type II fibres during longer periods of detraining (1 year) in elderly individuals (54). Then, in the same study after a subsequent period of retraining there was a significant increase in myonuclear number, yet in type II fibres only. These findings therefore demonstrated an exaggerated response for myonuclei accrual during retraining in type-II fibres perhaps due to earlier training, albeit the initial training itself did not evoke a significant average increase in myonuclei nor a retention of myonuclei following detraining across the entire group. This suggests, in an elderly population, that the significant loss of myonuclei during the detraining period also could provide a stimulus for the enhanced myonuclear accretion during later retraining and this response preferentially occurred in type II muscle fibres in elderly individuals. Consequently, this study was unable to determine whether there was a positive memory of training in elderly human skeletal muscle due to the lack of response during the initial training period, however, was able to demonstrate enhanced myonuclear accrual after encountering a negative stimulus of detraining.

Overall, there are very few human studies and those that have been conducted contain few participants, therefore demonstrating large variability in the myonuclear responses to training. Subsequently, this has made it difficult to firmly conclude whether a cellular muscle memory occurs in human skeletal muscle. Furthermore, these studies highlight there may be different myonuclear response to training, detraining, and retraining between young and elderly individuals. Despite this, a recent meta-analysis encompassing all the currently available human exercise and myonuclei-related studies detected a significant 9% increase in myonuclei number after training. However, as suggested above, due to the low participant numbers coupled with the heterogenous responses to training, it was not possible to detect whether a retention of myonuclei during detraining exists (48). Therefore, larger human studies across age are required in the future to conclude whether human muscle possesses a cellular memory of earlier training.

Limitations of experimental models and cross species comparisons in muscle cell memory research

The discrepancies in myonuclei accretion across rodent and human studies alluded to in the recent meta-analysis (48) suggests there is a 2.6 magnitude larger response in the accrual of new myonuclei after loading stimuli in rodents (+23%) compared to after training stimuli in humans (+9%). One explanation may be that typical rodent models of exercise-induced hypertrophy are often more extreme models amongst more genetically similar rodents compared to resistance exercise in

humans with a varied genetic background, leading to heterogenous hypertrophic and associated myonuclei response. Therefore, whether myonuclear accretion and retention is due to a true cell memory mechanism or influenced more strongly by loading procedure influencing the magnitude of hypertrophy, the rate of myonuclear accretion between species, or the heterogeneous of response in humans, is yet to be fully unravelled. The same meta-analysis also demonstrated that rodents can lose 6.6% of their myonuclei during detraining after gaining 23% in response to initial training period (48), suggestive that some, but not all myonuclei are retained after training in rodents. Furthermore, atrophic stimuli in rodents causes a loss of myonuclei at different rates across divergent muscle groups. Where the gastrocnemius and plantaris lose their myonuclei after atrophy, other muscles that are more resistant to muscle loss stimuli such as the soleus do not lose their myonuclei, perhaps further suggestive that fibre type can influence the extent to which myonuclei are retained or lost (48). Most human studies are exclusively undertaken in the vastus lateralis muscle of the quadriceps. Therefore, it is difficult to assess whether myonuclear loss or retention varies across muscles composed of different fibre type proportions. Therefore, a deeper investigation of the fibre type-specific changes in myonuclei in humans is warranted. Finally, research investigating whether myonuclei are lost or retained during atrophy is highly controversial, casting further complexity on the interpretation of myonuclear retention and loss.

What happens to myonuclei if they are lost during atrophy?

Studies that have used synergistic ablation to evoke hypertrophy in rodent EDL muscle have demonstrated that increases in myonuclei number are retained and not lost during a subsequent period of muscle atrophy caused by denervation (44). Despite these interesting findings, these data have been criticised for the use of denervation as the model of muscle loss, where removing or severing the nerve is a severe way of inducing rapid atrophy, where in recent meta-analyses satellite cell number has been demonstrated to either decrease or increase in response denervation in divergent rodent muscle groups (48). For example, the EDL and TA muscle may increase satellite cell content compared with other muscles such as the soleus muscle that reduce their satellite cell number in response to atrophy (48). Therefore, at least in the EDL muscle, it is perhaps difficult to distinguish whether myonuclei have been retained from the overload period or whether the denervation has helped keep satellite cell number high, and therefore helped maintain myonuclear number. Despite this, a study using both denervation (nerve severing) and tetrodotoxin (TTX)-induced atrophy (a model of disuse via release of TTX neurodetoxin to the nerves that silence neural input to the hindlimb muscles), both demonstrated no loss of myonuclei using a sophisticated and direct observation technique of single fibre lapse imaging (55). Using TUNEL labelling these studies suggested there was no apoptosis in myonuclei, that was already theoretically problematic to hypothesise in multinucleated fibres, as nuclei would have to undergo programmed cell death without cell disintegration. This was further supported in the same study, that demonstrated there was apoptosis in single celled populations of stomal and satellite cells in response to denervation and TTX-induced atrophy, but not in myonuclei (55). Presumably because these types of single cells can

undergo programmed cell death and cell disintegration. Therefore, these later studies support the notion that myonuclei are not lost and that they can be retained even during atrophy. However, this remains highly controversial and is debated extensively in the literature (56-60), with recent studies suggesting that myonuclear apoptosis can occur after hindlimb unloading in rodents via elevated DNase X in myonuclei leading to DNA fragmentation and degradation (61). It may also be logical to propose that the alterations in myonuclei following a period of disuse atrophy maybe quite different than those occurring after a period of detraining following an earlier period of training induced growth and myonuclear accrual. Therefore, whilst the understanding of myonuclear loss following disuse is important for understanding the mechanisms of inactivity induced atrophy, perhaps this process is not as relevant for the study of cellular muscle memory after growth, especially compared to the effect of myonuclear retention/loss during periods of detraining induced muscle atrophy after earlier training periods. However, the loss of nuclei after a period of atrophy may be relevant for muscle memory in instances where repeated encounters with muscle wasting stimuli occur. Indeed, repeated muscle wasting is a clinically relevant issue, especially in elderly populations, where after an injury and consequent disuse, individuals lose muscle and become weaker and are therefore more likely to suffer a repeated fall injury that may result in further muscle loss in the future (62). Repeated falling is then more highly associated with earlier morbidity and mortality. Therefore, whether a negative memory via loss of myonuclei after an earlier encounter with atrophy, that are perhaps not regained during recovery, leads to a greater loss of muscle mass when the same or similar atrophic stimulus occurs again later warrants future investigation.

Summary of cellular muscle memory

Collectively, research to date suggests that anabolic stimuli such as testosterone, mechanical overload and resistance exercise mimicking stimuli in animal models can promote the acquisition of new myonuclei, most of which may be retained (except progressive weighted wheel running) and therefore may serve to enable an enhanced response to the same or similar hypertrophic stimulus in the future. Therefore, there is evidence for cellular muscle memory in most rodent models, especially after non-physiological hypertrophy. However, it seems that there is still some controversy as to whether human muscle retains myonuclei after physiologically relevant load-induced growth such as resistance training. This is probably due to the limited human intervention studies coupled with the varied response between individuals, populations and muscle groups that have been investigated thus far. Therefore, additional training, detraining, retraining as well as atrophy, recovery, and repeated atrophy studies across larger and varied human populations in different muscles and/or fibre-types are required to conclude whether a cellular muscle memory exists in humans.

Theories for the mechanistic understanding of muscle memory continued

Epigenetic muscle memory: An introduction

519 The first to study to suggest that human skeletal muscle tissue possessed an epigenetic memory of
520 resistance exercise-induced muscle growth was undertaken by our group in 2018 (4). The study
521 investigated how DNA in human skeletal muscle was epigenetically modified (via DNA methylation)
522 during a period of resistance training followed by detraining and a subsequent period of retraining. In
523 this study, the DNA retained some of its epigenetic modifications after an earlier period of training,
524 even into the detraining period when training was completely ceased, and lean mass returned to pre-
525 training levels. Furthermore, some of the genes that demonstrated these retained epigenetic
526 signatures also displayed a larger magnitude of change in DNA methylation levels with later
527 retraining, suggestive that the earlier training period ´primed´ DNA for an enhanced response after
528 later retraining. Furthermore, these changes were also associated with the largest enhancements in
529 transcript expression of the same genes after later retraining that corresponded to the largest
530 increases in lean muscle mass (**Figure 2**). Overall, this study was the first to demonstrate that human
531 skeletal muscle retained epigenetic modifications from earlier training that persisted into the detraining
532 and retraining periods, and this was associated with enhancements in gene expression during later
533 retraining. Studies have also replicated similar findings after training, detraining and retraining in
534 rodents (6, 63) and aged humans (7), further supporting that skeletal muscle has an epigenetic
535 memory of exercise across species and into older age.
536
537 The conceptualisation of these initial studies and the role of epigenetics in muscle memory came from
538 the wider cell biology and epigenetic fields. This included observations that muscle derived cells once
539 isolated *in vitro* retain characteristics from the *in vivo* niche in which they were derived. To the authors
540 knowledge, this was first shown by Professor Claire Stewart's group, where primary skeletal muscle
541 derived cells isolated from cancer patients exhibited inappropriate cellular proliferation compared with
542 healthy non-cancerous aged-matched controls (64). Since these studies, the retention of cellular
543 characteristics from the *in vivo* niche after isolation *in vitro* have also been confirmed in the physically
544 active, diabetic, obese and elderly (reviewed in (2)). In 2016, we undertook an *in-vitro* study to
545 investigate whether epigenetic muscle memory existed as a proof of principle. To do this, we exposed
546 proliferating C2C12 muscle cells to an acute stimulus of high dose of Tumour Necrosis Factor alpha
547 (TNF-α), previously been observed to evoke myotube atrophy *in-vitro*, and then performed serial
548 passaging where the cells underwent 30 population doublings with no exogenous TNF-α present. The
549 cells were then subjected to a later proliferative life exposure of TNF-α and allowed to differentiate (8).
550 We observed, the cells that received the acute early proliferative life exposure to TNF-α were more
551 susceptible to impaired differentiation and myotube formation upon the second later life exposure
552 compared to relevant controls that had not received the early life exposure (8). Therefore, the cells
553 seemed to retain a memory, at least at the 'morphological' level, of an earlier encounter with
554 inflammation. Therefore, we became interested in the mechanisms by which this 'morphological'
555 memory may occur. Meanwhile, the field of epigenetics within molecular biology was rapidly
556 expanding and data was emerging from undernutrition studies *in-utero* and the resulting changes in
557 skeletal muscle of the offspring as well as epidemiological studies in humans demonstrating low birth
558 weight also having impairments in muscle mass and function into older age. It therefore became a

559 working hypothesis that environmental encounters experienced throughout life could be contributing
560 to these observations and this could be due to epigenetic modifications, reviewed in (2, 65). We
561 therefore investigated epigenetic modification of DNA methylation in our *in-vitro* muscle cell model
562 demonstrating retained morphological memory of inflammatory cytokine TNF-α (8). Indeed, we
563 demonstrated the cells that had received the early life dose of TNF-α exhibited hypermethylation of
564 the crucial myogenic regulatory factor, myoD, that was then retained for 30 cellular divisions.
565 Indicating that the cells 'remembered' an early acute dose (48-hour exposure) of TNF-α when
566 exposed to the same cytokine in later life. This was then associated with increased and retained
567 hypermethylation for 30 cellular divisions in a gene important for differentiation and myotube formation
568 (8). These data therefore served as a proof of principle for retained epigenetic imprints in muscle cells
569 over an extended period of time, and therefore an epigenetic muscle memory. These observations
570 thus laid platform for investigation for the role of epigenetics in skeletal muscle memory. However,
571 what was unknown at that time was whether human skeletal muscle *in-vivo* retained epigenetic
572 modifications such as DNA methylation after either negative stimuli (muscle wasting) or positive
573 stimuli (such as exercise).
574
575 Therefore, in the remaining sections of this review on muscle memory, we will first explain what
576 epigenetics is and summarise why it is important within the context of skeletal muscle adaptation and
577 muscle memory. We will then provide an overview of the studies investigating epigenetic muscle
578 memory and discuss how both the cellular and epigenetic theories of muscle memory may be
579 integrated, and work together. Finally, this review will focus on future directions and the wider
580 implications for muscle memory within the exercise and muscle biology field.
581
582 *What is epigenetics?*
583
584 Literally translated, 'epi' in Greek means 'on top' of genetics. Its origins can be traced to Conrad
585 Waddington in the 1940's who provided some of the first insights that helped define the term
586 epigenetics. He reported that there was a close interaction between environmental encounters and
587 underlying genetics to create a specific phenotype. For a modern day understanding of epigenetics,
588 the field describes how our tissues and cells are exposed to various environmental encounters (e.g.,
589 diet, physical activity, alcohol, drugs) that interact with our inherited DNA sequence (without altering
590 the sequence itself) to alter whether genes are turned on or off and to what extent this results in a
591 change in phenotype. A clear example of epigenetics in action comes from identical twin studies
592 (same inherited DNA), where one twin may be physically inactive and have a poor diet (environment)
593 compared with their more active twin sibling who consumes a healthier balanced diet. Their inherited
594 DNA sequence (genotype) is the same, however, they display very different phenotypes. Indeed,
595 epigenome-wide studies on discordant twins (those who lived together as children but separated as
596 adults) with different physical activity levels report vastly different epigenetic profiles in buccal cells
597 (66). Such varied epigenetic profiles and the resultant phenotypic changes are caused by biochemical
598 modifications on our cell's chromatin, histones (proteins that encase the DNA) or the DNA itself,

599 leading to either a more 'permissive' or 'repressive' state for gene expression to occur. These
600 modifications involve adding or removing small chemical groups such as acetyl or methyl groups to
601 histones or methyl groups to DNA and thus alter how accessible gene regulatory regions are for
602 transcription factors to bind DNA and regulate gene expression. These epigenetic modifications can
603 be dynamic and alter gene expression transiently (67) and therefore are able to return to basal levels
604 relatively quickly. However, importantly for epigenetic memory, some of these modifications can also
605 be retained longer periods, and some modifications can even be 'enhanced' if they have been
606 encountered before. Where, alluded to above, the epigenetic modification of DNA methylation in
607 human skeletal muscle has been shown to be retained during detraining and retraining following an
608 earlier resistance training period (4).
609
610 But what are these epigenetic modifications? Currently, more than 200 modifications and their
611 accompanying enzymes have been identified (68, 69), and the potential biochemical regulation of how
612 these epigenetic modifications may occur in muscle following exercise have been reviewed elsewhere
613 (70). The most common epigenetic modifications include: SUMOylation, phosphorylation,
614 ubiquitination, acetylation, and methylation. The latter two modifications are the most studied in
615 molecular biology, with acetylation (or deacetylation) as well as methylation (or demethylation)
616 occurring on histones, and methylation occurring on DNA itself. In this review, DNA methylation will
617 be the epigenetic modification of focus, as there have been no studies to date investigating retained
618 methylation or acetylation/deacetylation imprints on histones in field of skeletal muscle memory.
619
620 *What is DNA methylation and how does it occur?*
621
622 DNA methylation is the biochemical attachment of a covalent methyl (CH_3) group to the 5^{th} position of
623 the pyrimidine ring of a cytosine (C) nucleotide, resulting in 5-methylcytosine (5mC). This typically
624 occurs on the cytosine (C) nucleotide where it is followed by a guanine nucleotide (G) that is
625 separated by a phosphate ('p') group within the same strand of DNA. Therefore, this position is called
626 a CpG site. Approximately 1% of the human genome contains CpG sites with approximately 28 million
627 CpG sites over the entirety of our DNA (71, 72). Clusters of CpG sites within a short chromosomal
628 region are called CpG islands, and these occur more frequently in gene regulatory regions such as
629 promoters, enhancers and silencing regions involved in the initiation, activation, or suppression of
630 gene expression respectively (73). The addition or increase in CH_3 methyl groups to CpG sites when
631 investigating changes in methylation (termed ´differential methylation´), is commonly referred to as
632 ´hyper´-methylation. In contrast the removal of the CH_3 methyl group from cytosine nucleotides results
633 in 5-hydromethylcytosine (5-hmC) and therefore demethylation, also known as ´hypo´-methylation.
634 Specific enzymes are required to promote, maintain, or remove methyl groups and are referred to as
635 writers, readers and erasers respectively (reviewed in (70)). It's the DNA methyltransferases
636 (DNMTs), specifically DNMT3a, DNMT3b and DNMT1 that promote DNA methylation or
637 hypermethylation (74). Where DNMT3a and 3b are important for *de novo* or 'new' methylation, and
638 DNMT1 is responsible for maintaining methylation during cell division (75, 76). Alternatively, enzymes

known as the ten-eleven translocation (TET) enzymes (TET1, 2 and 3) remove methyl groups and are therefore responsible for demethylation or hypomethylation.

Why do DNA methylation modifications lead to changes in gene expression?

DNA methylation leads to changes in gene expression when the presence of CpG methylation recruits CpG methyl binding proteins that inhibit the transcriptional machinery (including transcription factors and RNA polymerase) and subsequently blocks gene transcription (77). Further, the presence of methylation can lead to the recruitment of proteins that leads to a tightening of the adjacent chromatin that impairs the transcriptional process (78). Whereas, removal of methylation causes a loosening of the adjacent chromatin, enabling gene expression to occur. This is most prominent if DNA methylation occurs in promoter or enhancer regions of the gene, where CpG islands are more prevalent. Therefore, this means that there is more likely to be (albeit not exclusively) an inverse relationship between methylation status and gene expression, where increased methylation leads to reduced gene expression and reduced methylation leads to increased gene expression. It is important to note however, that the opposite correspondent trend (positive association) between methylation and gene expression is more likely if methylation occurs in gene silencing regions. Furthermore, the complexity of DNA methylation really becomes apparent, when gene body methylation in non-regulatory regions has also been associated with altered transcription of the corresponding genes (79-81), where intragenic 5mC correlates with transcriptional strength (82). However, high methylation in these regions can also slow down elongation (83). Recent studies have also demonstrated that the presence of intragenic methylation (that is dependent on DNMT3b activity) protects the gene body from spurious entry of RNA polymerase II and inappropriate initiation of transcription. Therefore, this helps transcriptional fidelity via crosstalk between DNA methylation and histone H3K36 methylation (84). Conversely, TET3, which demethylates DNA via oxidizing 5-methylcytosine to 5-hydroxymethylcytosine (5hmC) can also prevent inappropriate intragenic entry of RNA polymerase II into highly expressed genes, therefore allowing continued transcription initiation at canonical start sites (85). Finally, DNA methylation can also regulate alternative splicing by impacting the inclusion of exons (86), thus perhaps also influencing the levels of transcript variants and ultimately protein isoforms.

DNA methylation and epigenetic memory in human skeletal muscle

To investigate whether young human skeletal muscle has an epigenetic memory of exercise-induced muscle growth, genome-wide DNA methylation (methylome) analysis (850K CpG sites) of whole human muscle tissue derived from the vastus lateralis (quadriceps) muscle was performed after 7 weeks of resistance training (3 days/week), 7 weeks of detraining (where participants returned to normal habitual physical activity) and another 7 weeks of training (or retraining) (4, 87). Targeted gene expression analysis of the genes that had the most significantly differentially methylated positions (DMPs) on CpG sites enabled validation of the DNA methylation data at the gene

679 expression level. Both gene expression and DNA methylation was also undertaken after an acute
680 resistance exercise bout in the same participants before they subsequently completed the training,
681 detraining and retraining intervention. It became immediately apparent that resistance exercise was a
682 predominantly hypomethylating stimulus across the genome after an acute bout of exercise (4),
683 something that had previously been demonstrated in candidate genes after acute endurance exercise
684 (88), yet not after resistance training and confirmed across the methylome. Subsequent analyses has
685 also demonstrated similar findings across the methylome after acute and chronic resistance exercise
686 in humans (4, 89-91), after synergist ablation (in rodents) (92), acute high intensity/sprint interval
687 exercise in humans (93), and after acute and chronic aerobic exercise in humans (10, 88). Indeed,
688 after chronic resistance training, we demonstrated that the number of hypomethylated DMPs were
689 greater than the number of hypermethylated DMPs after the first period of 7 weeks of resistance
690 training, where there were also significant increases in lower limb lean mass. Interestingly, the
691 number of hypo and hypermethylated positions remained similar even during detraining when
692 exercise was ceased, and lean muscle mass returned to pre-training (baseline) levels. Subsequently,
693 after the later retraining period, the number of hypomethylated DMPs more than doubled compared to
694 the earlier training period that was associated with the same magnitude (a doubling) increase in lean
695 mass after retraining compared with the earlier period of training (4). This was suggestive of an
696 enhanced number of CpG sites that were hypomethylated after later retraining as consequence of
697 having undertaken an earlier period of training. This enhancement in the number of hypomethylated
698 DNA sites after later retraining has now also been confirmed in a separate cohort of young adults
699 after a period of resistance training, cast immobilisation and retraining as well as in elderly individuals
700 after training, detraining and retraining (7). Following this initial analysis, the most significant DMPs
701 and their distinct temporal signatures over the period of training, detraining, and retraining were
702 assessed. Two distinct temporal methylation signatures identified suggested there was an epigenetic
703 memory of these genes. The first signature included genes that were hypomethylated after the initial
704 encounter with training-induced hypertrophy, where these CpG sites retained a hypomethylated
705 profile even during complete exercise cessation and lean mass returning to pre-training levels during
706 the detraining period (**Figure 2**). Furthermore, hypomethylation of these genes then continued
707 throughout retraining, where some genes even possessed enhanced hypomethylation after retraining
708 compared with earlier training. At the gene expression level, a subset of these genes (AXIN1, GRIK2,
709 CAMK4, TRAF1) also demonstrated increased gene expression after the first period of training.
710 Fascinatingly, during detraining where hypomethylation was retained, gene expression also remained
711 elevated, despite the participants undertaking no exercise and lean leg mass returning to pre-training
712 levels. Then in the retraining period, the genes with continuing and increased hypomethylation also
713 demonstrated enhanced gene expression (4) (**Figure 2**). Therefore, this gene profile indicated that
714 human muscle possessed an epigenetic memory at the DNA methylation level of previous training-
715 induced growth due to the retention of DNA methylation throughout detraining that led to larger
716 hypomethylation and enhanced gene expression after later retraining in this subset of genes. The
717 second temporal epigenetic and gene expression signature in another subset of genes (UBR5,
718 RPL35a, HEG1, PLA2G16, SETD3) also demonstrated hypomethylation and enhanced gene

719 expression following training. However, in opposition to the first temporal profile defined above, both
720 DNA methylation and gene expression returned to baseline levels during detraining. Therefore, there
721 was no retained hypomethylation or gene expression levels during this period. Despite this, these
722 genes then displayed an even greater hypomethylation during later retraining compared to the earlier
723 training period and further enhancements in gene expression after retraining compared to the initial
724 training (4) (**Figure 2**). This also suggested that due to being hypomethylated during the earlier
725 training (even despite returning to baseline methylation levels during detraining) these genes
726 demonstrated even greater hypomethylation during retraining. Finally, genes GRIK2, TRAF1, BICC1,
727 STAG1 demonstrated hypomethylation even after a single acute bout of resistance exercise, and then
728 retained this hypomethylation after the entire 21-week intervention with these genes demonstrating
729 largest enhancements in gene expression during the retraining period. These CpG sites and genes
730 were therefore dynamically and sensitively altered even after a single bout of resistance exercise and
731 were maintained for a long period of approximately 5 months (4).
732 One of the limitations of this work was that gene expression was assessed at the targeted and not
733 transcriptome-wide level that would otherwise enable the identification of all genes that changed at
734 the epigenetic as well as the transcriptomic level. To address this, the epigenetic memory profiles
735 were overlapped with gene expression changes across the human transcriptome (89). By integrating
736 over 110 and 181 publicly available human skeletal muscle transcriptomes after both acute and
737 chronic resistance training, respectively, this study was able to identify the degree of overlap between
738 the methylome and transcriptome and run a more integrated pathway analysis indicating the gene
739 pathways that were enriched for both differential methylation and gene expression. From these
740 genes, the number that demonstrated retained methylation during detraining and enhanced gene
741 expression during later retraining were predicted. A larger proportion of approximately 31% and 32%
742 of the upregulated genes at the transcriptome level after acute and chronic RE respectively, were
743 hypomethylated compared with 23% and 22.7% of genes that were downregulated and
744 hypermethylated after acute and chronic RE, respectively (89). Pathway analysis suggested that the
745 genes with differential methylation and altered gene expression were enriched in both methylome and
746 transcriptome data for many growth-related pathways related to matrix and actin structure/function
747 and remodelling, mechano-transduction, including PTK2/Focal Adhesion Kinase/FAK and
748 Phospholipase D (following chronic resistance exercise only), TGF-beta signalling and protein
749 synthesis (GSK3B after acute resistance exercise only), all of which have important functions in
750 skeletal muscle mass regulation (89). Other studies have also confirmed that an acute bout of
751 resistance exercise in trained men evoked enrichment of differential methylation in similar pathways
752 of focal adhesion, PI3K-Akt signalling, TGF-beta and MAPK signalling (90) as well as the mTOR
753 pathway after synergist ablation in mice (92). Resistance training, detraining and retraining in elderly
754 individuals also led to enriched methylation and gene expression in similar pathways (7). Indeed,
755 Sexton et al., (90) were able to provided more insights into the temporal regulation of alterations in
756 DNA methylation that precedes corresponding alterations in gene expression. To achieve this, the
757 authors analysed the methylome and transcriptome at both 3 and 6 hours post an acute resistance
758 exercise bout. At the pathway level there was a more concordant overlap of the same genes with the

3 hour DNA methylation changes and the gene expression at 6 hours, compared with DNA methylation and gene expression investigated at the same time point of 3 hrs or 6 hours (90). Suggesting there was a more prominent degree of overlap between the genes demonstrating methylation changes at 3 hours that also exhibit alterations in transcription at 6 hours after an acute bout of resistance exercise. Finally, by integrating the methylome and publicly available transcriptome after acute and chronic resistance exercise in the study discussed above (89). We were able to track the genes that were differentially methylated and expressed after acute and chronic resistance exercise across the genome and then identify which of these genes exhibited an epigenetic muscle memory profile after resistance training, detraining, and retraining. This led to the identification of an additional 51 genes that were differentially methylated and expressed after acute and chronic resistance exercise and training, respectively (89), and importantly that also demonstrated significantly altered methylation into the detraining and retraining periods (4). With 5 of these genes: FLNB, MYH9, SRGAP1, SRGN and ZMIZ1 demonstrating the temporal epigenetic memory profile as defined above (4), that of; retained hypomethylation into detraining after earlier training. From these 5 genes, Filamin B (FLNB) was increased at the gene expression level after acute and chronic resistance exercise and remained elevated after detraining and retraining where the gene remained as hypomethylated even during exercise cessation (detraining) (89). Increases in Filamin B expression were only statistically significant after the acute resistance exercise, perhaps due to the lack of RNA from all subjects meaning a smaller subset of participants from the original study could be analyzed. Despite this, the role of Filamin B requires more attention given the role of the filamin gene family as actin crosslinkers, and that Filamin A and C have been investigated following endurance and resistance exercise, respectively (94, 95), as well as being associated with autophagy in skeletal muscle (95). Overall, there is limited information regarding the role of Filamin B in the regulation of muscle mass and given its retained hypomethylation profile, Filamin B requires attention to confirm its role in epigenetic muscle memory.

Role of the identified epigenetic memory genes

Despite the discovery of the genes that have epigenetic memory profiles, the characterisation of these genes in the regulation of muscle memory after exercise-induced hypertrophy remain largely undetermined. To begin to unravel the deeper mechanisms of epigenetic muscle memory, one gene, UBR5, that was identified from the initial studies (4) has been investigated in more detail. UBR5 was a gene that demonstrated hypomethylation after training, where methylation returned to baseline levels during detraining and displayed enhanced hypomethylation and gene expression after retraining in human skeletal muscle (4). This previously uncharacterised gene in muscle was of particular interest, as UBR5 is an E3 Ubiquitin ligase that was positively correlated with increasing lean leg mass after training, detraining and retraining in humans as depicted in the second temporal profile described above (**see Figure 2**) (4). E3 Ubiquitin ligases have well-defined roles of 'tagging' proteins for degradation in the proteosome are therefore typically associated with skeletal muscle atrophy and not hypertrophy. For example, the well-characterised protein degradative E3 Ubiquitin ligases, MuRF1

and MAFbx, are primarily involved in degradation of muscle-specific proteins across a wide range of atrophy conditions, reviewed recently in (96). Therefore, the characterisation of UBR5 in skeletal muscle mass regulation required attention due to its associated role in hypertrophy and muscle memory. Indeed, further studies confirmed the role of this gene as a positive regulator of skeletal muscle hypertrophy and recovery from atrophy across various mammalian species *in vivo* and *in vitro,* as well as having an alternate regulation to MuRF1 and MAFbx after hypertrophy stimuli (97). To further confirm its mechanistic role, muscle-specific knock-down of UBR5 using electroporation of UBR5 RNAi in mice was undertaken that resulted in atrophy (98). This further confirmed that the presence of UBR5 is important for maintaining muscle mass. Other data in humans has also suggested that the A alleles of the rs10505025 and rs4734621 SNPs that affect the expression of the UBR5 gene, according to the Gene Tissue Expression (GTEX) project, were strongly associated with larger fast-twitch muscle fibres and strength/power performance versus endurance status in athletes (97). Therefore, again pointing to an important role for UBR5 in regulating muscle mass potentially in the larger faster fibres associated with greater athletic performance. Importantly, the same epigenetic memory profile, as described above for UBR5 (in the same UBR5 CpG site) was also confirmed in skeletal muscle within an independent study of young adults after a period of resistance training, cast immobilisation and later retraining (7). Overall, these data suggest that UBR5, first identified as an epigenetic muscle memory gene in human muscle, is an important regulator of muscle mass and indicates that there is a more complex interplay within the family of Ubiquitin E3 ligases, where despite their protein degradative role (e.g., MuRF1 and MAFbx), a subset of these ligases may also play a role in positively regulating muscle mass. Whilst speculative, this maybe because E3 ubiquitin ligases, such as UBR5, may instead destabilise proteins considered to be negative regulators of muscle mass such as PP2Ac (98). Despite these advances and insights so far, it is still necessary to mechanistically confirm the role of UBR5 in muscle memory by experimental manipulation (i.e., knockdown or overexpression) of this gene in a model of training, detraining and retraining to confirm its role in muscle memory.

Epigenetic muscle memory in aging skeletal muscle

An epigenetic memory has now also been demonstrated in aged human muscle tissue, where a similar model of 12 weeks training, detraining, and retraining has been undertaken in elderly individuals whilst investigating both the methylome and transcriptome in comparison with young adults undergoing a period of 12 resistance training, 2 cast immobilisation and a further 12 weeks of resistance retraining (7). In this study, there was a larger number of hypomethylated versus hypermethylated sites after both training periods with an even greater hypomethylated profile after retraining versus the initial training period across both young and old muscle (7), the same profile observed previously in young adult human muscle (4). Interestingly however, there was a greater hypermethylation after 12-week detraining in the elderly and 2 weeks cast immobilization in young muscle (7), that was different to the similar number of hypermethylated and hypomethylated sites after 7 weeks detraining in young adults in the earlier study (4). One explanation for this, could be that

2 weeks cast immobilization was a more aggressive negative insult in young adult muscle and a longer 12-week detraining period in older muscle was perhaps already on a background of hypermethylation (99), thus perhaps evoking even greater hypermethylation compared to 7 weeks detraining in young muscle (4). This latest study in young and elderly muscle also identified two interesting genes with respect to epigenetic muscle memory that displayed retained epigenetic profiles during the detraining/cast immobilisation periods. In the older men after training, the Vinculin (VCL) gene within the focal adhesion pathway (a pathway that also demonstrates enriched differential methylation after resistance exercise (4, 89, 90)), was hypomethylated after training, and then retained a hypomethylated during immobilisation and retraining, with the largest increase in gene expression occurring after retraining (7). Therefore, Vinculin tracked one of the same temporal epigenetic memory profiles identified in earlier studies described above (4) (**Figure 2**). At the protein level, Vinculin is an important structural component of the costamere (100), and connects the sarcomere to the cell membrane to stabilize muscle fibres during contraction. It is also crucial for mechano-transduction (101, 102) and is increased at the muscle gene expression level following chronic stimulation and disuse (103). It therefore appears focal adhesion pathway genes enriched at the differential methylation level after resistance training and retraining, including VCL (and FLNB discussed above), demonstrate an epigenetic memory. Given these genes roles in mechano-sensing and muscle mass regulation, their enhanced hypomethylation and gene expression during retraining maybe closely linked to the larger increases in muscle mass observed during later retraining. A second gene, AMOTL1, also demonstrated retained hypomethylation after training into immobilisation in younger men with enhanced hypomethylation into retraining (7). Importantly, this temporal response of AMOTL1 confirmed the same profile previously identified in younger men after resistance training, detraining, and retraining (4). AMOTL1 is important for activating YAP1 (an important gene within the mechano-sensitive YAP/TAZ pathway) enabling activation and proliferation of satellite cells (104). Therefore, AMOTL1 has now been confirmed as an epigenetic memory gene in two independent studies in human skeletal muscle and has a pre-established role in skeletal muscle, therefore should be investigated further for its mechanistic role in regulating epigenetic muscle memory.

Is there an epigenetic muscle memory to different types of exercise?

To date, epigenetic muscle memory has only been observed after resistance exercise. With aerobic training, detraining and retraining unable to demonstrate retained transcript expression levels during 9 months of detraining after an initial 3 months of aerobic training (105). Therefore, studies employing a similar study design to investigate genome-wide epigenetic modifications after aerobic training, detraining, and retraining are warranted in future human studies. Given the impact of high-intensity aerobic exercise and high-intensity/sprint interval exercise on promoting hypomethylation compared with lower intensity exercise (88, 93), it maybe that exercise intensity has an important role in stimulating alterations in DNA methylation (reviewed in (70)). Indeed, in collaboration with our group, unpublished data from Simone Porcelli's laboratory and primary researcher Andrea Pilotto,

879 investigated if there is an epigenetic memory of high-intensity exercise training. These unpublished
880 observations suggest there were 1,190 CpG sites in the human genome that demonstrate a retained
881 hypomethylated profile into detraining after a high intensity training period (Pilotto A et al,.
882 unpublished). Furthermore, the number of hypomethylated DMPs remained elevated during detraining
883 and retraining, whereas hypermethylation returned to baseline level in both conditions. Sepcifically,
884 the genes: *ADAM19, INPP5a, MTHFD1L, PDGFB, CAPN2* and *SLC16A3* as were identified as
885 hypomethylated genes with enhanced expression after initial training that maintained their
886 hypomethylated status and enhanced transcription even during detraining, indicating an epigenetic
887 memory of these genes' methylation signatures following earlier training (Pilotto A et al., unpublished).
888 The genes demonstrating an epigenetic memory were associated with metabolic pathways, calcium
889 signaling, lactate and pyruvate transport and mitochondrial enzymes. Overall, these data suggest that
890 there was an epigenetically regulated transcriptional memory of earlier high intensity training. These
891 data may also lead to a working hypothesis that there may be a mitochondrial epigenetic memory in
892 muscle. Where studies in the future should perhaps also investigate the mitochondrial DNA (mtDNA)
893 methylome after a period of training, detraining, and retraining. Indirect support for this hypothesis
894 exists in a recent study that demonstrated resistance exercise can remodel the mtDNA methylome
895 (106), and given the nature of the stimulus, it is plausible to suggest that aerobic and/or high intensity
896 exercise may perhaps achieve a more substantial epigenetic response in mtDNA, something that
897 requires attention in the near future.
898 *Epigenetic muscle memory across species*
899
900 Consistent with the findings in epigenetic muscle memory across young and elderly humans
901 discussed above, a recent investigation explored the methylation signatures using a model of high
902 intensity weighted wheel running training, detraining, retraining in rodents and reported a greater
903 hypomethylation and retention of DNA methylation signatures into detraining following an earlier
904 training period (6). What was exciting in these mice studies, is that the authors investigated the
905 methylome specifically of the myonuclei that resides directly within the muscle fibre and were able to
906 compare this with the methylome of other nuclei present in whole muscle tissue (termed interstitial
907 nuclei given these nuclei are located outside of the muscle fibre), after a period of 8 weeks training
908 (via PoWeR), detraining, and another 4 weeks of retraining (6). This study has advanced the field
909 and will probably be crucial in developing our understanding of how epigenetic memory in muscle is
910 integrated with the cellular muscle memory mechanism. Therefore, in the next section we discuss this
911 research in more detail to enable relevant future directions in this field.
912
913 *Integration of epigenetic and cellular muscle memory theories*
914
915 As alluded to directly above, in the epigenetic muscle memory studies in mice, the authors
916 investigated methylation signatures, specifically of the myonuclei compared with other interstitial
917 nuclei residing in the mouse plantaris muscle (6). To do this, they employed progressive weighted
918 wheel running (PoWeR) training in mice for 8 weeks, followed by 12 weeks of detraining and 4 weeks

of retraining. The group undertook reduced representation bisulfite sequencing (RRBS) and RNA-sequencing for genome-wide methylation and gene expression, respectively (6). Following the first 8-week training intervention there was slightly larger hypomethylation compared with hypermethylation in the DNA of the myonuclei, similar to that observed after initial training in humans (4, 7). However, in the interstitial nuclei, there was a greater a predominance of hypomethylation versus hypermethylation compared to the myonuclei. The enrichment of methylation in specific pathways was also different between the myonuclei and interstitial nuclei in these rodents. Where one of the most enriched pathways was the Wnt signalling pathway, with Wnt related genes demonstrating hypomethylation within the myonuclei, compared with hypermethylation in promoter regions within the interstitial cell nuclei (6). These alterations in differential methylation enriched in similar pathways between nuclei types resident in skeletal muscle tissue suggests that there may be an important epigenetic role for different cell types in skeletal muscle tissue in response to exercise training. Single nuclei analysis of DNA methylation profiles therefore seem important to determine in human muscle to investigate whether epigenetic muscle memory resides in both the nuclei of muscle fibres themselves as well as the nuclei of non-muscle cell types that contribute to the skeletal muscle niche. One caveat may be that it has been reported approximately 40-60% of nuclei in mouse muscle are myonuclei, with human muscle containing more like 80%+ myonuclei compared with other nuclei types (92, 107). Therefore, the contribution of epigenetic alterations in divergent nuclei from different cell types in human muscle is potentially not as strong as that observed in mouse muscle. However, it is clear that future studies need to determine the relative contribution of differential methylation between differing nucleus types in human muscle after resistance exercise to be able to quantify this appropriately and draw conclusions.

It is also important to highlight that in published human data after training, both acute and chronic resistance exercise growth-related pathways demonstrate enriched hypomethylation (4, 89, 90). While Wnt signalling did not seem to be significantly enriched in humans after resistance exercise (4), in unpublished observations, Wnt signalling in the DNA from a muscle biopsy homogenate was enriched with hypomethylation after high intensity training in humans (Pilloto A et al., Unpublished), corroborating the hypomethylation observed in the DNA from myonuclei after PoWeR training in mice. Where, PoWeR training is perhaps more similar to high intensity exercise than heavy resistance exercise in humans (108), and therefore this unpublished human data potentially support the role for enriched hypomethylation in the Wnt signalling pathway observed in mouse myonuclei.

Returning to the above rodent study, after a period of detraining in mice, the authors were able to also identify that DNA methylation signatures altered after the initial PoWeR training period were retained, indicating an epigenetic memory in muscle following training in mice (6). Therefore, this study further substantiates cross-species evidence for an epigenetic muscle memory that was first observed in human muscle (4, 7). Interestingly, in these mouse studies, hypomethylation was also maintained throughout detraining in the interstitial nuclei and hypermethylation was predominantly retained in the myonuclei (92). This indicated that the hypomethylation that persisted in the DNA in human muscle

959 homogenates, aligned with the trend observed in the mouse interstitial nuclei versus the myonuclei.
960 Having said this, the authors also reported retention of hypomethylation in the myonuclei, albeit with
961 fewer genes than the number of those that were hypermethylated. Furthermore, we also identified
962 retained hypermethylation in the muscle homogenate in humans, just the predominant profile was that
963 of retained hypomethylation (4). The proportion of myonuclei to interstitial nuclei in human versus
964 mice studies, described above, may also play a role in these differences observed between species.
965 However, these intricacies across species require further investigation to further tease out the nuclei-
966 specific alterations in human muscle and their relative contribution to muscle memory.
967
968 Finally, it is important to note that in these repeated training studies in mice, the authors did not report
969 the DNA methylome or transcriptome analyses after later retraining (6). Therefore, the authors were
970 unable to test the existence of the second epigenetic memory profile to repeated training as described
971 above in humans (4) (**Figure 2**). Specifically, whether initial hypomethylation after training is further
972 enhanced leading to a greater level of hypomethylation during later retaining (4), something that
973 would be important to corroborate in mice studies in the future. Nevertheless, these studies in mice
974 have advanced the field and highlight the need for further analysis of cell-specific epigenetic
975 responses for understanding epigenetic memory in skeletal muscle. In the future, it would also be
976 important to determine the methylation of nuclei from different cell types making up the interstitial
977 component. Given that in the study design above, satellite cell-derived myonuclei would also be in the
978 interstitial fraction (6). Therefore, given that satellite cells may also be incorporated into the muscle
979 fibres to become myonuclei following training, this means that the epigenetic alterations in satellite
980 cells following training, detraining and retaining have not yet been distinguished. Earlier work by the
981 same group have attempted to determine methylome differences between myonuclei versus
982 myonuclei plus satellite cells using genetic mouse models (109). Here the authors were able to
983 fluorescently tag myonuclei with high specificity before and following PoWeR training. Using this
984 approach, myonuclei residing in the muscle at the start of training and that persisted during the
985 training period were fluorescently labelled and compared to all the post-training myonuclear pool that
986 included both resident myonuclei present at the start of training plus satellite cell-derived myonuclei
987 acquired from the training period. Therefore, the contribution of satellite cell-derived myonuclei
988 induced alterations in the methylome could therefore be, in-part, deduced by comparing these two
989 groups. One caveat using this design means that it is difficult to separate absolute changes in the
990 myonuclear methylome versus satellite cells alone. However, thus far this is the best attempt using a
991 novel and exciting genetic mouse model to investigate the satellite cell methylome. The authors
992 identified that myonuclear DNA was hypomethylated after PoWeR training and this hypomethylation
993 was enriched in gene promoters involved in growth-related pathways and protein turnover, whereas
994 the myonuclear plus satellite cell methylome profiles were shifted towards transcription factor
995 regulation and cell-cell signalling. Further, by comparing myonuclei-specific methylation profiles to
996 already published single-nucleus transcriptional analysis from the same conditions, suggested that
997 satellite cell-derived myonuclei may preferentially provide specific ribosomal proteins to muscle fibres
998 and therefore could provide a memory of these genes to the growing muscle fibre. Interestingly,

methylation in ribosomal protein gene, Rpl35a, was identified as hypomethylated in the myonuclei plus satellite cell condition as well as increased at the gene expression level relative to the myonuclei only condition after PoWeR training in mice. Importantly, this gene was also identified as having an epigenetic memory profile in human skeletal muscle tissue (109). Taken together, these data across human and rodent studies suggest that an epigenetic memory of specific genes involved in different processes may come from distinct cell nucleus types within the muscle niche (**Figure 3**). For example, growth-related epigenetic memory genes from myonuclei compared with transcription factor, cell-cell signalling and ribosomal protein genes from satellite cell nuclei. In addition to these existing studies attempting to investigate satellite cell specific methylome profiles and muscle memory, there has been some recent DNA methylation data from isolated satellite cells that suggests that epigenetic retention of DNA methylation can occur specifically in this cell type after muscle injury and therefore may impact adaptation of muscle into adulthood (110). Something we will discuss in more detail in the following section.

Overall, the determination of epigenetic profiles of both activated, fused and retained satellite cells versus already resident myonuclei could be important for defining the integration of both the cellular and epigenetic memory theories of skeletal muscle adaptation to exercise stimuli. However, many challenges to this work in humans arise from the low number of satellite cells in a muscle tissue biopsy (~1-5% of total nuclei in human muscle (111, 112)), and therefore genetic mouse models and human derived cell cultures will most likely be needed to investigate the contribution of epigenetic memory that resides in satellite cells themselves. However, as alluded to above, satellite cells are potentially an important source of epigenetically retained imprints given they can be activated and proliferate, thus may pass their epigenetic information to their daughter cells that can then fuse to the existing muscle fibres when encountering growth stimuli. Overall, we propose there may be a synergistic interaction between the myonuclei accrued (cellular memory theory) and a specific epigenetic contribution from both the myonuclei and satellite cells themselves (epigenetic memory theory) that in turn may be intertwined with epigenetic changes in other cell types within the muscle tissue niche. For example, it is known that even in the absence of fusion, satellite cells can communicate with other cell types such as interstitial fibrogenic cells, as well as the muscle fibres themselves (40, 41). Therefore, studies investigating the integration of both the cellular and epigenetic components of muscle memory in different cell types within the muscle tissue niche will provide a very fruitful avenue for future discovery in the field of muscle memory.

Is there a negative epigenetic memory in skeletal muscle?

So far, the focus of the review has addressed whether skeletal muscle possesses a memory of positive stimuli. However, it is also possible that a ‘negative’ memory may also exist in skeletal muscle. For example, an encounter with a ‘negative’ muscle wasting or atrophic stimuli such as disuse or immobilisation caused by an injury or hospitalisation may result in the muscle becoming more susceptible to muscle wasting if the same/similar wasting stimuli is encountered again in the

future. It is therefore also possible that muscle may possess a 'negative' memory after muscle loss associated with metabolic disease or cancer. Indeed, as summarised in the introduction, studies demonstrate that muscle cells remember negative encounters once isolated *in-vitro,* such as impaired lipid metabolism and insulin sensitivity, when cells are obtained from T2D and obese individuals, or uncontrolled cell growth in cells isolated from cancer patients (reviewed in (2, 9)). Furthermore, evidence from undernutrition *in-utero* in animal models and epigenetic changes skeletal muscle of the offspring demonstrate that early life *in-utero* encounters with growth restricting environments can negatively alter muscle size, fibre type and function of the offspring's muscle (reviewed in (2, 9). With recent evidence that maternal exercise mediates epigenetic changes in the offspring at both the DNA methylation (113) and histone level (114).

Proof of concept for a negative muscle memory exists from *in-vitro* experiments, where muscle cells retain an epigenetic signature of an early proliferative life encounter with a muscle wasting dose of inflammation (high dose TNF-α to levels experienced in muscle wasting conditions such as cancer cachexia) (8). Consequently, when TNF-α is encountered later in the muscle cells proliferative lifespan they demonstrate an increased susceptibility to reductions in differentiation and myotube growth (8). In a most recent study in mice, there is also evidence for an epigenetic memory of negative stimuli from muscle injury in earlier life having a long-lasting epigenetic impact via DNA methylation in muscle stem cells after normal development of mature muscle. Interestingly, having an earlier life muscle injury meant there were CpG sites that also retained their demethylated (hypomethylated) status in mature muscle from the earlier injury even when accounting for epigenetic changes that occur during normal development (110). While the muscle injury caused predominantly hypomethylation in gene regions, transcriptome analysis suggested a down regulation of the transcripts associated with the methylation changes. Using Tet3 knockdown, the authors were also able to suggest that the changes in demethylation were necessary for muscle stem cell activation and proper regeneration (110). In these studies, there was not a repeated later muscle injury, so while the authors hypothesised that earlier injury could 'prime' the muscle for later adaptation, this was not investigated. Having said this, these data seem to suggest a negative encounter of muscle injury (multiple needle stick injury in rodents) may have a positive effect on later regeneration and in this scenario may not fit into the definition of 'negative´ muscle memory and notion that muscle becomes more susceptible to muscle wasting in later life if it has been encountered earlier. Based on these data, it is perhaps logical to hypothesise that a positive or negative memory to muscle wasting periods is likely to be highly dependent on the severity of the negative encounter of injury or disuse, disease state and age of the individual. While this recent study sheds light on early life encounters with negative stimuli (the injury protocol was a severe model of multiple needle stick injury in mice) there is currently no publisded study on negative memory from earlier wasting periods in humans caused by physiologically relevant stimuli such as injury or disuse, and how these encounters impact the ability of the muscle to respond to the same encounter in later life. This is especially prudent, for example, in the elderly, where if these individuals encounter a fall and muscle injury, they will lose muscle mass and function, and are then more likely to encounter a repeated fall injury and repeated muscle

wasting, with repeated encounters with wasting associated with earlier frailty, morbidity, and mortality (62). Identifying gene targets involved in negative muscle memory may therefore help identify those genes that could be targeted to provide a therapy. A strategy that could provide a clinically relevant time window would be after an elderly person falls and loses muscle mass. Where, if known, memory genes would be targeted (e.g., via gene therapy) in the recovery period following this accident, with the idea that targeting these genes could protect the muscle if it was to encounter another fall injury and a repeated period of muscle wasting in the future. Recent unpublished observations from our group suggest that muscle may possess a negative memory in some but not all muscles, as well as loss of strength that may be more indicative of a negative neural memory. In this unpublished study, young healthy humans (male and female) undertook 2 weeks of disuse-induced atrophy via unilateral limb immobilization, followed by 7 weeks of recovery then another 2 weeks of atrophy (referred to as repeated atrophy). We found there were comparable reductions in total leg lean mass (-3%) and mCSA of the VL (-9%) after atrophy and repeated atrophy. However, a loss of mCSA of the rectus femoris only occurred after repeated atrophy (-3%), coupled with a greater loss of muscle strength compared to the initial atrophy period (Turner D et al., unpublished). Ongoing myonuclei number, methylome and transcriptomic analyses will seek to determine whether a cellular or epigenetic memory of earlier and repeated atrophy exists.

Finally, to the authors knowledge there is only one study that has assessed, albeit indirectly, whether a negative epigenetic memory in human skeletal muscle exists, whereby breast cancer patients demonstrated retained DNA methylation profiles over 10 years after diagnosis and treatment compared to relevant aged-matched controls (10). However, so far there is very little direct *in-vivo* evidence in adult humans of a negative epigenetic muscle memory in skeletal muscle tissue and therefore this paradigm requires future investigation perhaps using the design of repeated muscle wasting stimuli with a period of recovery between these stimuli, as discussed above. These studies are perhaps difficult to justify ethically in elderly individuals, and therefore will probably require aged animal and or *in-vitro* experimentation in elderly derived muscle cells to progress this area.

Can exercise reset the epigenetic DNA methylome after negative encounters?

It has been demonstrated that aged human skeletal muscle tissue and muscle derived cells demonstrate hypermethylation of DNA compared with young adult skeletal muscle tissue (7, 99, 111) and that this hypermethylation occurs in gene regulatory regions (115, 116). Yet, in a recent large scale meta-analysis of aging muscle across the genome, there was a predominance of hypomethylation across the genome (117). Despite this, it is still perhaps the case that the hypermethylation trend observed in aged muscle occurs in gene regulatory regions such as promoter regions (115, 116) and more studies need to investigate, not just promoter and enhancer regions, but also silencer regions, as well as intragenic and gene body methylation and determine whether the methylation and expression levels are correlated in aged skeletal muscle. Interestingly, the same meta-analysis suggested that 68% of the differentially expressed genes in aged skeletal are reduced

(117). This would support that overtime the significant DNA methylation changes, perhaps an accumulation of DNA methylation in gene regulatory regions leads to an overall suppression of gene expression in skeletal muscle with age. However, only 68 of the same genes significantly altered at the DNA methylation level demonstrated corresponding changes at the expression level with age in skeletal muscle (117), perhaps suggesting a small, yet important number of epigenetically modified genes result in changes corresponding to gene expression in aged skeletal muscle. Even given this recent knowledge, when these DNA methylation changes occur across the lifespan are not entirely clear. However, given the potential 'accumulation' of epigenetic marks in aged muscle and that exercise considerably remodels the DNA methylome landscape, as described above, it could be hypothesised that exercise could be epigenetically anti-aging. Also, perhaps due to epigenetic muscle memory, exercise could offset the impact of negative epigenetic events on skeletal muscle health during the lifespan.

To support this hypothesis, muscle taken from elderly individuals who have undertaken regular exercise throughout their life display a more hypomethylated DNA profile compared to elderly individuals who have been less active throughout their life (57). It has also been demonstrated that those who are aerobically physically active have inversely methylated signatures to those observed in aged muscle (99). Moreover, aging has been shown to cause hypermethylation to the mitochondrial (mtDNA) genome in skeletal muscle, where progressive resistance training was able to 'rejuvenate' the hypermethylated mtDNA signature towards a more hypomethylated state that was closely reminiscent of the signature observed in trained young adult muscle (94). This was therefore also suggestive that the negative epigenetic signatures associated with aging muscle might be counteracted by undertaking exercise training. Recent studies shed light on this hypothesis where exercise mitigates the negative effects of maternal high-fat consumption on the offspring (114), suggestive of a positive memory of exercise both before (in the offspring) or in later (aged) life. Similarly, resistance training and aerobic training have been shown to partially reset the skeletal muscle methylome of both aged individuals (7) and cancer survivors (10) back towards profiles observed in relevant healthy young and relevant healthy aged-matched controls respectively. In rodents, late life PoWeR exercise training (mice aged 22-24 months) prevents aged associated promoter hypermethylation and promotes a younger DNA methylation age by 8 weeks (8% of total lifespan) as measured by epigenetic clocks (118, 119). Finally, meta-analysis of over three thousand methylome and transcriptome samples from skeletal muscle has also been able to predict that for every additional unit of increasing VO_2 max with age is accompanied by a change in methylation equivalent to 1.6 years of "rejuvenation" of the age-related methylation changes (117). More simply, increased aerobic training into older age (that increases VO_2 max) will improve the epigenetic profile in muscle to those observed in younger profiles. In terms of muscle memory, there is currently no data studying individuals that have trained in the past and encounter muscle wasting stimuli to see if the memory from the earlier training can help protect the muscle from a wasting encounter. Also, it has not been directly studied how long epigenetic memory can last, however, it is intriguing to think that it

maybe be remembered for years after an initial stimulus (10), something that clearly warrants future study.

Future directions

It is not currently known how long cellular or epigenetic memory lasts and this question is likely to be the focus of future research in this area. It is also likely that other epigenetic modifications such as those that occur on histone proteins and other factors such as the three-dimensional chromatin configuration, could also be retained after exercise and lead to an enhanced genomic response to future exercise. Therefore, these other epigenetic modifications are also likely to be important areas of muscle memory research in the future.

Further, it is also interesting to hypothesise that by altering the exercise frequency, intensity, or timing of training this could make memory of exercise last longer. Initial data suggests that epigenetic alterations can be somewhat distinct between acute exercise at different intensities within aerobic, high intensity and resistance exercise (88, 90, 93), and therefore requires studies of chronic training, detraining, retraining to assess whether a certain intensity is advantageous to promote a longer lasting muscle memory. Hypothetically, addressing these important questions would allow the incorporation of this type of 'optimised muscle memory' training into an athletes or recreationally active persons periodised training programme. This may help reduce the total volume or intensity of training required, meaning more time could be spent recovering (therefore reducing injury) or on skill-specific tasks for a given event/sport. For example, if athletes or the public could train at higher volume/intensity less frequently and still have the same adaptation due to muscle memory this would be beneficial in enabling more time for recovery or enabling more time efficient exercise programs. However, more studies that are designed to test these specific exercise variables throughout training, detraining, and retraining are required to answer these questions.

Finally, understanding negative muscle memory will be important for identifying the genes associated with retained epigenetic profiles associated with muscle wasting. It is becoming clearer that exercise can help, at least partially reset the epigenetic landscape in the muscle of aged and diseased individuals. However, given that exercise may not always be possible in elderly, frail or diseased individuals, identifying gene targets involved in negative muscle memory may help identify future strategies of therapeutic intervention for muscle wasting.

Acknowledgments

Adam P. Sharples group are funded by The Research Council of Norway project number 314157. The graphical abstract figure and figures 1-3 were created with BioRender.com.

Conflicts of interest

The authors declare no conflicts of interest.

References

1. **Roubenoff R**. Sarcopenia and its implications for the elderly. *European Journal of Clinical Nutrition* 54: S40-S47, 2000.
2. **Sharples AP, Stewart CE, and Seaborne RA**. Does skeletal muscle have an 'epi'-memory? The role of epigenetics in nutritional programming, metabolic disease, aging and exercise. *Aging cell* 15: 603-616, 2016.
3. **Staron RS, Leonardi MJ, Karapondo DL, Malicky ES, Falkel JE, Hagerman FC, and Hikida RS**. Strength and skeletal muscle adaptations in heavy-resistance-trained women after detraining and retraining. *J Appl Physiol (1985)* 70: 631-640, 1991.
4. **Seaborne RA, Strauss J, Cocks M, Shepherd S, O'Brien TD, van Someren KA, Bell PG, Murgatroyd C, Morton JP, Stewart CE, and Sharples AP**. Human Skeletal Muscle Possesses an Epigenetic Memory of Hypertrophy. *Scientific reports* 8: 1898, 2018.
5. **Egner IM, Bruusgaard JC, Eftestøl E, and Gundersen K**. A cellular memory mechanism aids overload hypertrophy in muscle long after an episodic exposure to anabolic steroids. *The Journal of Physiology* 591: 6221-6230, 2013.
6. **Wen Y, Dungan CM, Mobley CB, Valentino T, von Walden F, and Murach KA**. Nucleus Type-Specific DNA Methylomics Reveals Epigenetic "Memory" of Prior Adaptation in Skeletal Muscle. *Function* 2: 2021.
7. **Blocquiaux S, Ramaekers M, Van Thienen R, Nielens H, Delecluse C, De Bock K, and Thomis M**. Recurrent training rejuvenates and enhances transcriptome and methylome responses in young and older human muscle. *JCSM Rapid Communications* 5: 10-32, 2022.
8. **Sharples AP, Polydorou I, Hughes DC, Owens DJ, Hughes TM, and Stewart CE**. Skeletal muscle cells possess a 'memory' of acute early life TNF-alpha exposure: role of epigenetic adaptation. *Biogerontology* 17: 603-617, 2016.
9. **Sharples AP, and Seaborne RA**. Chapter Ten - Exercise and DNA methylation in skeletal muscle. In: *Sports, Exercise, and Nutritional Genomics*, edited by Barh D, and Ahmetov IIAcademic Press, 2019, p. 211-229.
10. **Gorski PP, Raastad T, Ullrich M, Turner DC, Hallén J, Savari SI, Nilsen TS, and Sharples AP**. Aerobic exercise training resets the human skeletal muscle methylome 10 years after breast cancer treatment and survival. *Faseb j* 37: e22720, 2023.
11. **Goldberg AL**. Work-induced growth of skeletal muscle in normal and hypophysectomized rats. *Am J Physiol* 213: 1193-1198, 1967.
12. **Heron MI, and Richmond FJR**. In-series fiber architecture in long human muscles. *Journal of Morphology* 216: 35-45, 1993.
13. **Katz B**. How cells communicate. *Sci Am* 205: 209-220, 1961.
14. **Mauro A**. Satellite cell of skeletal muscle fibers. *J Biophys Biochem Cytol* 9: 493-495, 1961.
15. **Gayraud-Morel B, Chrétien F, Flamant P, Gomès D, Zammit PS, and Tajbakhsh S**. A role for the myogenic determination gene Myf5 in adult regenerative myogenesis. *Dev Biol* 312: 13-28, 2007.
16. **Ustanina S, Carvajal J, Rigby P, and Braun T**. The Myogenic Factor Myf5 Supports Efficient Skeletal Muscle Regeneration by Enabling Transient Myoblast Amplification. *Stem cells (Dayton, Ohio)* 25: 2006-2016, 2007.
17. **Yin H, Price F, and Rudnicki MA**. Satellite cells and the muscle stem cell niche. *Physiol Rev* 93: 23-67, 2013.
18. **Yablonka-Reuveni Z, Rudnicki MA, Rivera AJ, Primig M, Anderson JE, and Natanson P**. The transition from proliferation to differentiation is delayed in satellite cells from mice lacking MyoD. *Dev Biol* 210: 440-455, 1999.
19. **Sabourin LA, Girgis-Gabardo A, Seale P, Asakura A, and Rudnicki MA**. Reduced differentiation potential of primary MyoD-/- myogenic cells derived from adult skeletal muscle. *J Cell Biol* 144: 631-643, 1999.
20. **Cornelison DD, and Wold BJ**. Single-cell analysis of regulatory gene expression in quiescent and activated mouse skeletal muscle satellite cells. *Dev Biol* 191: 270-283, 1997.
21. **Berkes CA, and Tapscott SJ**. MyoD and the transcriptional control of myogenesis. *Seminars in cell & developmental biology* 16: 585-595, 2005.
22. **McKay BR, Ogborn DI, Bellamy LM, Tarnopolsky MA, and Parise G**. Myostatin is associated with age-related human muscle stem cell dysfunction. *Faseb j* 26: 2509-2521, 2012.

23. **Nederveen JP, Joanisse S, Séguin CM, Bell KE, Baker SK, Phillips SM, and Parise G**. The effect of exercise mode on the acute response of satellite cells in old men. *Acta physiologica (Oxford, England)* 215: 177-190, 2015.
24. **Petrella JK, Kim JS, Mayhew DL, Cross JM, and Bamman MM**. Potent myofiber hypertrophy during resistance training in humans is associated with satellite cell-mediated myonuclear addition: a cluster analysis. *J Appl Physiol (1985)* 104: 1736-1742, 2008.
25. **Verdijk LB, Gleeson BG, Jonkers RA, Meijer K, Savelberg HH, Dendale P, and van Loon LJ**. Skeletal muscle hypertrophy following resistance training is accompanied by a fiber type-specific increase in satellite cell content in elderly men. *J Gerontol A Biol Sci Med Sci* 64: 332-339, 2009.
26. **Bellamy LM, Joanisse S, Grubb A, Mitchell CJ, McKay BR, Phillips SM, Baker S, and Parise G**. The Acute Satellite Cell Response and Skeletal Muscle Hypertrophy following Resistance Training. *PLoS ONE* 9: e109739, 2014.
27. **Borowik AK, Davidyan A, Peelor FF, Ill, Voloviceva E, Doidge SM, Bubak MP, Mobley CB, McCarthy JJ, Dupont-Versteegden EE, and Miller BF**. Skeletal Muscle Nuclei in Mice are not Post-mitotic. *Function* 4: 2022.
28. **Rosenblatt JD, and Parry DJ**. Gamma irradiation prevents compensatory hypertrophy of overloaded mouse extensor digitorum longus muscle. *J Appl Physiol (1985)* 73: 2538-2543, 1992.
29. **McCarthy JJ, Mula J, Miyazaki M, Erfani R, Garrison K, Farooqui AB, Srikuea R, Lawson BA, Grimes B, Keller C, Van Zant G, Campbell KS, Esser KA, Dupont-Versteegden EE, and Peterson CA**. Effective fiber hypertrophy in satellite cell-depleted skeletal muscle. *Development* 138: 3657-3666, 2011.
30. **Egner IM, Bruusgaard JC, and Gundersen K**. Satellite cell depletion prevents fiber hypertrophy in skeletal muscle. *Development* 143: 2898-2906, 2016.
31. **Egner IM, Bruusgaard JC, and Gundersen K**. An apparent lack of effect of satellite cell depletion on hypertrophy could be due to methodological limitations. Response to 'Methodological issues limit interpretation of negative effects of satellite cell depletion on adult muscle hypertrophy'. *Development* 144: 1365-1367, 2017.
32. **McCarthy JJ, Dupont-Versteegden EE, Fry CS, Murach KA, and Peterson CA**. Methodological issues limit interpretation of negative effects of satellite cell depletion on adult muscle hypertrophy. *Development* 144: 1363-1365, 2017.
33. **Hansson K-A, Eftestøl E, Bruusgaard JC, Juvkam I, Cramer AW, Malthe-Sørenssen A, Millay DP, and Gundersen K**. Myonuclear content regulates cell size with similar scaling properties in mice and humans. *Nature Communications* 11: 6288, 2020.
34. **Cramer AAW, Prasad V, Eftestøl E, Song T, Hansson K-A, Dugdale HF, Sadayappan S, Ochala J, Gundersen K, and Millay DP**. Nuclear numbers in syncytial muscle fibers promote size but limit the development of larger myonuclear domains. *Nature Communications* 11: 6287, 2020.
35. **Englund DA, Figueiredo VC, Dungan CM, Murach KA, Peck BD, Petrosino JM, Brightwell CR, Dupont AM, Neal AC, Fry CS, Accornero F, McCarthy JJ, and Peterson CA**. Satellite Cell Depletion Disrupts Transcriptional Coordination and Muscle Adaptation to Exercise. *Function* 2: 2020.
36. **Bagley JR, Denes LT, McCarthy JJ, Wang ET, and Murach KA**. The myonuclear domain in adult skeletal muscle fibres: past, present and future. *The Journal of Physiology* 601: 723-741, 2023.
37. **Fry CS, Lee JD, Jackson JR, Kirby TJ, Stasko SA, Liu H, Dupont-Versteegden EE, McCarthy JJ, and Peterson CA**. Regulation of the muscle fiber microenvironment by activated satellite cells during hypertrophy. *Faseb j* 28: 1654-1665, 2014.
38. **Goh Q, and Millay DP**. Requirement of myomaker-mediated stem cell fusion for skeletal muscle hypertrophy. *eLife* 6: e20007, 2017.
39. **Goh Q, Song T, Petrany MJ, Cramer AAW, Sun C, Sadayappan S, Lee S-J, and Millay DP**. Myonuclear accretion is a determinant of exercise-induced remodeling in skeletal muscle. *eLife* 8: e44876, 2019.
40. **Murach KA, Peck BD, Policastro RA, Vechetti IJ, Van Pelt DW, Dungan CM, Denes LT, Fu X, Brightwell CR, Zentner GE, Dupont-Versteegden EE, Richards CI, Smith JJ, Fry CS, McCarthy JJ, and Peterson CA**. Early satellite cell communication creates a permissive environment for long-term muscle growth. *iScience* 24: 102372, 2021.
41. **Fry CS, Kirby TJ, Kosmac K, McCarthy JJ, and Peterson CA**. Myogenic Progenitor Cells Control Extracellular Matrix Production by Fibroblasts during Skeletal Muscle Hypertrophy. *Cell stem cell* 20: 56-69, 2017.
42. **Petrella JK, Kim JS, Mayhew DL, Cross JM, and Bamman MM**. Potent myofiber hypertrophy during resistance training in humans is associated with satellite cell-mediated myonuclear addition: a cluster analysis. *J Appl Physiol* 104: 1736-1742, 2008.

43. **Snijders T, Aussieker T, Holwerda A, Parise G, van Loon LJC, and Verdijk LB**. The concept of skeletal muscle memory: Evidence from animal and human studies. *Acta physiologica (Oxford, England)* 229: e13465, 2020.
44. **Bruusgaard JC, Johansen IB, Egner IM, Rana ZA, and Gundersen K**. Myonuclei acquired by overload exercise precede hypertrophy and are not lost on detraining. *Proc Natl Acad Sci U S A* 107: 15111-15116, 2010.
45. **Murach KA, Mobley CB, Zdunek CJ, Frick KK, Jones SR, McCarthy JJ, Peterson CA, and Dungan CM**. Muscle memory: myonuclear accretion, maintenance, morphology, and miRNA levels with training and detraining in adult mice. *Journal of cachexia, sarcopenia and muscle* 11: 1705-1722, 2020.
46. **Lee H, Kim K, Kim B, Shin J, Rajan S, Wu J, Chen X, Brown MD, Lee S, and Park JY**. A cellular mechanism of muscle memory facilitates mitochondrial remodelling following resistance training. *J Physiol* 596: 4413-4426, 2018.
47. **Eftestøl E, Ochi E, Juvkam IS, Hansson KA, and Gundersen K**. A juvenile climbing exercise establishes a muscle memory boosting the effects of exercise in adult rats. *Acta physiologica (Oxford, England)* 236: e13879, 2022.
48. **Rahmati M, McCarthy JJ, and Malakoutinia F**. Myonuclear permanence in skeletal muscle memory: a systematic review and meta-analysis of human and animal studies. *Journal of cachexia, sarcopenia and muscle* 13: 2276-2297, 2022.
49. **Kadi F, Schjerling P, Andersen LL, Charifi N, Madsen JL, Christensen LR, and Andersen JL**. The effects of heavy resistance training and detraining on satellite cells in human skeletal muscles. *J Physiol* 558: 1005-1012, 2004.
50. **Psilander N, Eftestøl E, Cumming KT, Juvkam I, Ekblom MM, Sunding K, Wernbom M, Holmberg H-C, Ekblom B, Bruusgaard JC, Raastad T, and Gundersen K**. Effects of training, detraining, and retraining on strength, hypertrophy, and myonuclear number in human skeletal muscle. *Journal of Applied Physiology* 126: 1636-1645, 2019.
51. **Murach KA, Dungan CM, Dupont-Versteegden EE, McCarthy JJ, and Peterson CA**. "Muscle memory" not mediated by myonuclear number? Secondary analysis of human detraining data. *Journal of Applied Physiology* 127: 1814-1816, 2019.
52. **Venturelli M, Schena F, Naro F, Reggiani C, Pereira Guimaraes M, de Almeida Costa Campos Y, Costa Moreira O, Fernandes da Silva S, Silva Marques de Azevedo PH, Dixit A, Srivastav S, Hinkley JM, Seaborne RA, Viggars M, Sharples AP, Mahmassani ZS, Drummond MJ, and Gondin J**. Commentaries on Viewpoint: "Muscle memory" not mediated by myonuclear number? Secondary analysis of human detraining data. *J Appl Physiol (1985)* 127: 1817-1820, 2019.
53. **Blocquiaux S, Gorski T, Van Roie E, Ramaekers M, Van Thienen R, Nielens H, Delecluse C, De Bock K, and Thomis M**. The effect of resistance training, detraining and retraining on muscle strength and power, myofibre size, satellite cells and myonuclei in older men. *Exp Gerontol* 133: 110860, 2020.
54. **Snijders T, Leenders M, de Groot LCPGM, van Loon LJC, and Verdijk LB**. Muscle mass and strength gains following 6 months of resistance type exercise training are only partly preserved within one year with autonomous exercise continuation in older adults. *Experimental Gerontology* 121: 71-78, 2019.
55. **Bruusgaard JC, and Gundersen K**. In vivo time-lapse microscopy reveals no loss of murine myonuclei during weeks of muscle atrophy. *J Clin Invest* 118: 1450-1457, 2008.
56. **Kirby TJ, and Dupont-Versteegden EE**. Cross Talk proposal: Myonuclei are lost with ageing and atrophy. *The Journal of Physiology* 600: 2077-2080, 2022.
57. **Kirby TJ, and Dupont-Versteegden EE**. Cross Talk rebuttal: Kirby and Dupont-Versteegden. *The Journal of Physiology* 600: 2085-2086, 2022.
58. **Schwartz L, and Gundersen K**. Cross Talk rebuttal: Schwartz and Gundersen. *The Journal of Physiology* 600: 2087-2088, 2022.
59. **Schwartz LM, and Gundersen K**. Cross Talk opposing view: Myonuclei do not undergo apoptosis during skeletal muscle atrophy. *The Journal of Physiology* 600: 2081-2084, 2022.
60. **Viggars MR, Owens DJ, Stewart C, Coirault C, Mackey AL, and Jarvis JC**. PCM1 labeling reveals myonuclear and nuclear dynamics in skeletal muscle across species. *American Journal of Physiology-Cell Physiology* 324: C85-C97, 2023.
61. **Yang H, Wang H, Pan F, Guo Y, Cao L, Yan W, and Gao Y**. New Findings: Hindlimb Unloading Causes Nucleocytoplasmic Ca2+ Overload and DNA Damage in Skeletal Muscle. *Cells* 12: 1077, 2023.
62. **Ambrose AF, Paul G, and Hausdorff JM**. Risk factors for falls among older adults: A review of the literature. *Maturitas* 75: 51-61, 2013.

63. **Sharples AP**. Skeletal Muscle Possesses an Epigenetic Memory of Exercise: Role of Nucleus Type-Specific DNA Methylation. *Function (Oxf)* 2: zqab047, 2021.
64. **Foulstone EJ, Savage PB, Crown AL, Holly JM, and Stewart CE**. Adaptations of the IGF system during malignancy: human skeletal muscle versus the systemic environment. *Horm Metab Res* 35: 667-674, 2003.
65. **Sharples AP, Seaborne RA, and Stewart CE**. Epigenetics of Skeletal Muscle Aging. In: *Epigenetics of Aging and Longevity*, edited by Vaiserman AM. Boston: Academic Press, 2018, p. 389-416.
66. **Duncan GE, Avery A, Thorson JLM, Nilsson EE, Beck D, and Skinner MK**. Epigenome-wide association study of physical activity and physiological parameters in discordant monozygotic twins. *Scientific reports* 12: 20166, 2022.
67. **Fisher AG, Seaborne RA, Hughes TM, Gutteridge A, Stewart C, Coulson JM, Sharples AP, and Jarvis JC**. Transcriptomic and epigenetic regulation of disuse atrophy and the return to activity in skeletal muscle. *Faseb j* 31: 5268-5282, 2017.
68. **Duan G, and Walther D**. The Roles of Post-translational Modifications in the Context of Protein Interaction Networks. *PLOS Computational Biology* 11: e1004049, 2015.
69. **Minguez P, Parca L, Diella F, Mende DR, Kumar R, Helmer-Citterich M, Gavin AC, van Noort V, and Bork P**. Deciphering a global network of functionally associated post-translational modifications. *Mol Syst Biol* 8: 599, 2012.
70. **Seaborne RA, and Sharples AP**. The Interplay Between Exercise Metabolism, Epigenetics, and Skeletal Muscle Remodeling. *Exerc Sport Sci Rev* 48: 188-200, 2020.
71. **Lister R, Pelizzola M, Dowen RH, Hawkins RD, Hon G, Tonti-Filippini J, Nery JR, Lee L, Ye Z, Ngo QM, Edsall L, Antosiewicz-Bourget J, Stewart R, Ruotti V, Millar AH, Thomson JA, Ren B, and Ecker JR**. Human DNA methylomes at base resolution show widespread epigenomic differences. *Nature* 462: 315-322, 2009.
72. **Ziller MJ, Gu H, Müller F, Donaghey J, Tsai LT, Kohlbacher O, De Jager PL, Rosen ED, Bennett DA, Bernstein BE, Gnirke A, and Meissner A**. Charting a dynamic DNA methylation landscape of the human genome. *Nature* 500: 477-481, 2013.
73. **Bird AP**. CpG-rich islands and the function of DNA methylation. *Nature* 321: 209-213, 1986.
74. **Suzuki MM, and Bird A**. DNA methylation landscapes: provocative insights from epigenomics. *Nat Rev Genet* 9: 465-476, 2008.
75. **Trasler J, Deng L, Melnyk S, Pogribny I, Hiou-Tim F, Sibani S, Oakes C, Li E, James SJ, and Rozen R**. Impact of Dnmt1 deficiency, with and without low folate diets, on tumor numbers and DNA methylation in Min mice. *Carcinogenesis* 24: 39-45, 2003.
76. **Denis H, Ndlovu MN, and Fuks F**. Regulation of mammalian DNA methyltransferases: a route to new mechanisms. *EMBO Rep* 12: 647-656, 2011.
77. **Bogdanović O, and Veenstra GJ**. DNA methylation and methyl-CpG binding proteins: developmental requirements and function. *Chromosoma* 118: 549-565, 2009.
78. **Jones PL, Veenstra GJ, Wade PA, Vermaak D, Kass SU, Landsberger N, Strouboulis J, and Wolffe AP**. Methylated DNA and MeCP2 recruit histone deacetylase to repress transcription. *Nature genetics* 19: 187-191, 1998.
79. **Anastasiadi D, Esteve-Codina A, and Piferrer F**. Consistent inverse correlation between DNA methylation of the first intron and gene expression across tissues and species. *Epigenetics & Chromatin* 11: 37, 2018.
80. **Ball MP, Li JB, Gao Y, Lee JH, LeProust EM, Park IH, Xie B, Daley GQ, and Church GM**. Targeted and genome-scale strategies reveal gene-body methylation signatures in human cells. *Nat Biotechnol* 27: 361-368, 2009.
81. **Brenet F, Moh M, Funk P, Feierstein E, Viale AJ, Socci ND, and Scandura JM**. DNA methylation of the first exon is tightly linked to transcriptional silencing. *PLoS ONE* 6: e14524, 2011.
82. **Jones PA**. The DNA methylation paradox. *Trends in Genetics* 15: 34-37, 1999.
83. **Veloso A, Kirkconnell KS, Magnuson B, Biewen B, Paulsen MT, Wilson TE, and Ljungman M**. Rate of elongation by RNA polymerase II is associated with specific gene features and epigenetic modifications. *Genome research* 24: 896-905, 2014.
84. **Neri F, Rapelli S, Krepelova A, Incarnato D, Parlato C, Basile G, Maldotti M, Anselmi F, and Oliviero S**. Intragenic DNA methylation prevents spurious transcription initiation. *Nature* 543: 72-77, 2017.
85. **Wu F, Li X, Looso M, Liu H, Ding D, Günther S, Kuenne C, Liu S, Weissmann N, Boettger T, Atzberger A, Kolahian S, Renz H, Offermanns S, Gärtner U, Potente M, Zhou Y, Yuan X, and Braun T**. Spurious transcription causing innate immune responses is prevented by 5-hydroxymethylcytosine. *Nature genetics* 2022.

86. **Shayevitch R, Askayo D, Keydar I, and Ast G**. The importance of DNA methylation of exons on alternative splicing. *Rna* 24: 1351-1362, 2018.
87. **Seaborne RA, Strauss J, Cocks M, Shepherd S, O'Brien TD, Someren KAV, Bell PG, Murgatroyd C, Morton JP, Stewart CE, Mein CA, and Sharples AP**. Methylome of human skeletal muscle after acute & chronic resistance exercise training, detraining & retraining. *Scientific data* 5: 180213, 2018.
88. **Barres R, Yan J, Egan B, Treebak JT, Rasmussen M, Fritz T, Caidahl K, Krook A, O'Gorman DJ, and Zierath JR**. Acute exercise remodels promoter methylation in human skeletal muscle. *Cell Metab* 15: 405-411, 2012.
89. **Turner DC, Seaborne RA, and Sharples AP**. Comparative Transcriptome and Methylome Analysis in Human Skeletal Muscle Anabolism, Hypertrophy and Epigenetic Memory. *Scientific Reports (Nature)* 9: 4251, 2019.
90. **Sexton CL, Godwin JS, McIntosh MC, Ruple BA, Osburn SC, Hollingsworth BR, Kontos NJ, Agostinelli PJ, Kavazis AN, Ziegenfuss TN, Lopez HL, Smith R, Young KC, Dwaraka VB, Frugé AD, Mobley CB, Sharples AP, and Roberts MD**. Skeletal Muscle DNA Methylation and mRNA Responses to a Bout of Higher Versus Lower Load Resistance Exercise in Previously Trained Men. *Cells* 12: 263, 2023.
91. **Rowlands DS, Page RA, Sukala WR, Giri M, Ghimbovschi SD, Hayat I, Cheema BS, Lys I, Leikis M, Sheard PW, Wakefield SJ, Breier B, Hathout Y, Brown K, Marathi R, Orkunoglu-Suer FE, Devaney JM, Leiken B, Many G, Krebs J, Hopkins WG, and Hoffman EP**. Multi-omic integrated networks connect DNA methylation and miRNA with skeletal muscle plasticity to chronic exercise in Type 2 diabetic obesity. *Physiological Genomics* 46: 747-765, 2014.
92. **Von Walden F, Rea M, Mobley CB, Fondufe-Mittendorf Y, McCarthy JJ, Peterson CA, and Murach KA**. The myonuclear DNA methylome in response to an acute hypertrophic stimulus. *Epigenetics : official journal of the DNA Methylation Society* 15: 1151-1162, 2020.
93. **Maasar MF, Turner DC, Gorski PP, Seaborne RA, Strauss JA, Shepherd SO, Cocks M, Pillon NJ, Zierath JR, Hulton AT, Drust B, and Sharples AP**. The Comparative Methylome and Transcriptome After Change of Direction Compared to Straight Line Running Exercise in Human Skeletal Muscle. *Front Physiol* 12: 619447, 2021.
94. **Deshmukh A, Coffey VG, Zhong Z, Chibalin AV, Hawley JA, and Zierath JR**. Exercise-induced phosphorylation of the novel Akt substrates AS160 and filamin A in human skeletal muscle. *Diabetes* 55: 1776-1782, 2006.
95. **Ulbricht A, Gehlert S, Leciejewski B, Schiffer T, Bloch W, and Hohfeld J**. Induction and adaptation of chaperone-assisted selective autophagy CASA in response to resistance exercise in human skeletal muscle. *Autophagy* 11: 538-546, 2015.
96. **Hughes DC, Baehr LM, Waddell DS, Sharples AP, and Bodine SC**. Ubiquitin Ligases in Longevity and Aging Skeletal Muscle. *International Journal of Molecular Sciences* 23: 7602, 2022.
97. **Seaborne RA, Hughes DC, Turner DC, Owens DJ, Baehr LM, Gorski P, Semenova EA, Borisov OV, Larin AK, Popov DV, Generozov EV, Sutherland H, Ahmetov, II, Jarvis JC, Bodine SC, and Sharples AP**. UBR5 is a novel E3 ubiquitin ligase involved in skeletal muscle hypertrophy and recovery from atrophy. *J Physiol* 597: 3727-3749, 2019.
98. **Hughes DC, Turner DC, Baehr LM, Seaborne RA, Viggars M, Jarvis JC, Gorski PP, Stewart CE, Owens DJ, Bodine SC, and Sharples AP**. Knockdown of the E3 ubiquitin ligase UBR5 and its role in skeletal muscle anabolism. *Am J Physiol Cell Physiol* 320: C45-c56, 2021.
99. **Turner DC, Gorski PP, Maasar MF, Seaborne RA, Baumert P, Brown AD, Kitchen MO, Erskine RM, Dos-Remedios I, Voisin S, Eynon N, Sultanov RI, Borisov OV, Larin AK, Semenova EA, Popov DV, Generozov EV, Stewart CE, Drust B, Owens DJ, Ahmetov, II, and Sharples AP**. DNA methylation across the genome in aged human skeletal muscle tissue and muscle-derived cells: the role of HOX genes and physical activity. *Scientific Reports (Nature)* 10: 15360, 2020.
100. **Pardo JV, Siliciano JD, and Craig SW**. A vinculin-containing cortical lattice in skeletal muscle: transverse lattice elements ("costameres") mark sites of attachment between myofibrils and sarcolemma. *Proc Natl Acad Sci U S A* 80: 1008-1012, 1983.
101. **Rindom E, and Vissing K**. Mechanosensitive Molecular Networks Involved in Transducing Resistance Exercise-Signals into Muscle Protein Accretion. *Front Physiol* 7: 547, 2016.
102. **Chorev DS, Volberg T, Livne A, Eisenstein M, Martins B, Kam Z, Jockusch BM, Medalia O, Sharon M, and Geiger B**. Conformational states during vinculin unlocking differentially regulate focal adhesion properties. *Scientific reports* 8: 2693, 2018.
103. **Rezvani M, Ornatsky OI, Connor MK, Eisenberg HA, and Hood DA**. Dystrophin, vinculin, and aciculin in skeletal muscle subject to chronic use and disuse. *Medicine and science in sports and exercise* 28: 79-84, 1996.

104. **Li L, and Fan CM**. A CREB-MPP7-AMOT Regulatory Axis Controls Muscle Stem Cell Expansion and Self-Renewal Competence. *Cell reports* 21: 1253-1266, 2017.
105. **Lindholm ME, Giacomello S, Werne Solnestam B, Fischer H, Huss M, Kjellqvist S, and Sundberg CJ**. The Impact of Endurance Training on Human Skeletal Muscle Memory, Global Isoform Expression and Novel Transcripts. *PLoS genetics* 12: e1006294, 2016.
106. **Ruple BA, Godwin JS, Mesquita PHC, Osburn SC, Vann CG, Lamb DA, Sexton CL, Candow DG, Forbes SC, Frugé AD, Kavazis AN, Young KC, Seaborne RA, Sharples AP, and Roberts MD**. Resistance training rejuvenates the mitochondrial methylome in aged human skeletal muscle. *Faseb j* 35: e21864, 2021.
107. **Bengtsen M, Winje IM, Eftestøl E, Landskron J, Sun C, Nygård K, Domanska D, Millay DP, Meza-Zepeda LA, and Gundersen K**. Comparing the epigenetic landscape in myonuclei purified with a PCM1 antibody from a fast/glycolytic and a slow/oxidative muscle. *PLoS genetics* 17: e1009907, 2021.
108. **Dungan CM, Murach KA, Frick KK, Jones SR, Crow SE, Englund DA, Jr. IJV, Figueiredo VC, Levitan BM, Satin J, McCarthy JJ, and Peterson CA**. Elevated myonuclear density during skeletal muscle hypertrophy in response to training is reversed during detraining. *American Journal of Physiology-Cell Physiology* 316: C649-C654, 2019.
109. **Murach KA, Dungan CM, Walden Fv, and Wen Y**. Epigenetic evidence for distinct contributions of resident and acquired myonuclei during long-term exercise adaptation using timed in vivo myonuclear labeling. *American Journal of Physiology-Cell Physiology* 322: C86-C93, 2022.
110. **Falick Michaeli T, Sabag O, Fok R, Azria B, Monin J, Nevo Y, Gielchinsky Y, Berman BP, Cedar H, and Bergman Y**. Muscle injury causes long-term changes in stem-cell DNA methylation. *Proc Natl Acad Sci U S A* 119: e2212306119, 2022.
111. **Zykovich A, Hubbard A, Flynn JM, Tarnopolsky M, Fraga MF, Kerksick C, Ogborn D, MacNeil L, Mooney SD, and Melov S**. Genome-wide DNA methylation changes with age in disease-free human skeletal muscle. *Aging cell* 13: 360-366, 2014.
112. **Mackey AL, Kjaer M, Charifi N, Henriksson J, Bojsen-Moller J, Holm L, and Kadi F**. Assessment of satellite cell number and activity status in human skeletal muscle biopsies. *Muscle & Nerve* 40: 455-465, 2009.
113. **Kusuyama J, Alves-Wagner AB, Conlin RH, Makarewicz NS, Albertson BG, Prince NB, Kobayashi S, Kozuka C, Møller M, Bjerre M, Fuglsang J, Miele E, Middelbeek RJW, Xiudong Y, Xia Y, Garneau L, Bhattacharjee J, Aguer C, Patti ME, Hirshman MF, Jessen N, Hatta T, Ovesen PG, Adamo KB, Nozik-Grayck E, and Goodyear LJ**. Placental superoxide dismutase 3 mediates benefits of maternal exercise on offspring health. *Cell Metab* 33: 939-956.e938, 2021.
114. **Kusuyama J, Makarewicz NS, Albertson BG, Alves-Wagner AB, Conlin RH, Prince NB, Alves CRR, Ramachandran K, Kozuka C, Xiudong Y, Xia Y, Hirshman MF, Hatta T, Nagatomi R, Nozik ES, and Goodyear LJ**. Maternal Exercise-Induced SOD3 Reverses the Deleterious Effects of Maternal High-Fat Diet on Offspring Metabolism Through Stabilization of H3K4me3 and Protection Against WDR82 Carbonylation. *Diabetes* 71: 1170-1181, 2022.
115. **Voisin S, Harvey NR, Haupt LM, Griffiths LR, Ashton KJ, Coffey VG, Doering TM, Thompson JM, Benedict C, Cedernaes J, Lindholm ME, Craig JM, Rowlands DS, Sharples AP, Horvath S, and Eynon N**. An epigenetic clock for human skeletal muscle. *Journal of cachexia, sarcopenia and muscle* 11: 887-898, 2020.
116. **Voisin S, Jacques M, Landen S, Harvey NR, Haupt LM, Griffiths LR, Gancheva S, Ouni M, Jähnert M, Ashton KJ, Coffey VG, Thompson JM, Doering TM, Gabory A, Junien C, Caiazzo R, Verkindt H, Raverdy V, Pattou F, Froguel P, Craig JM, Blocquiaux S, Thomis M, Sharples AP, Schürmann A, Roden M, Horvath S, and Eynon N**. Meta-analysis of genome-wide DNA methylation and integrative omics of age in human skeletal muscle. *Journal of cachexia, sarcopenia and muscle* 12: 1064-1078, 2021.
117. **Voisin S, Seale K, Jacques M, Landen S, Harvey NR, Haupt LM, Griffiths LR, Ashton KJ, Coffey VG, Thompson J-LM, Doering TM, Lindholm ME, Walsh C, Davison G, Irwin R, McBride C, Hansson O, Asplund O, Heikkinen AE, Piirilä P, Pietiläinen K, Ollikainen M, Blocquiaux S, Thomis M, Sharples AP, and Eynon N**. Exercise rejuvenates the skeletal muscle methylome and transcriptome in humans. *bioRxiv* 2022.2012.2027.522062, 2022.
118. **Murach KA, Dimet-Wiley AL, Wen Y, Brightwell CR, Latham CM, Dungan CM, Fry CS, and Watowich SJ**. Late-life exercise mitigates skeletal muscle epigenetic aging. *Aging cell* 21: e13527, 2022.
119. **Jones III RG, Dimet-Wiley A, Haghani A, da Silva FM, Brightwell CR, Lim S, Khadgi S, Wen Y, Dungan CM, Brooke RT, Greene NP, Peterson CA, McCarthy JJ, Horvath S, Watowich**

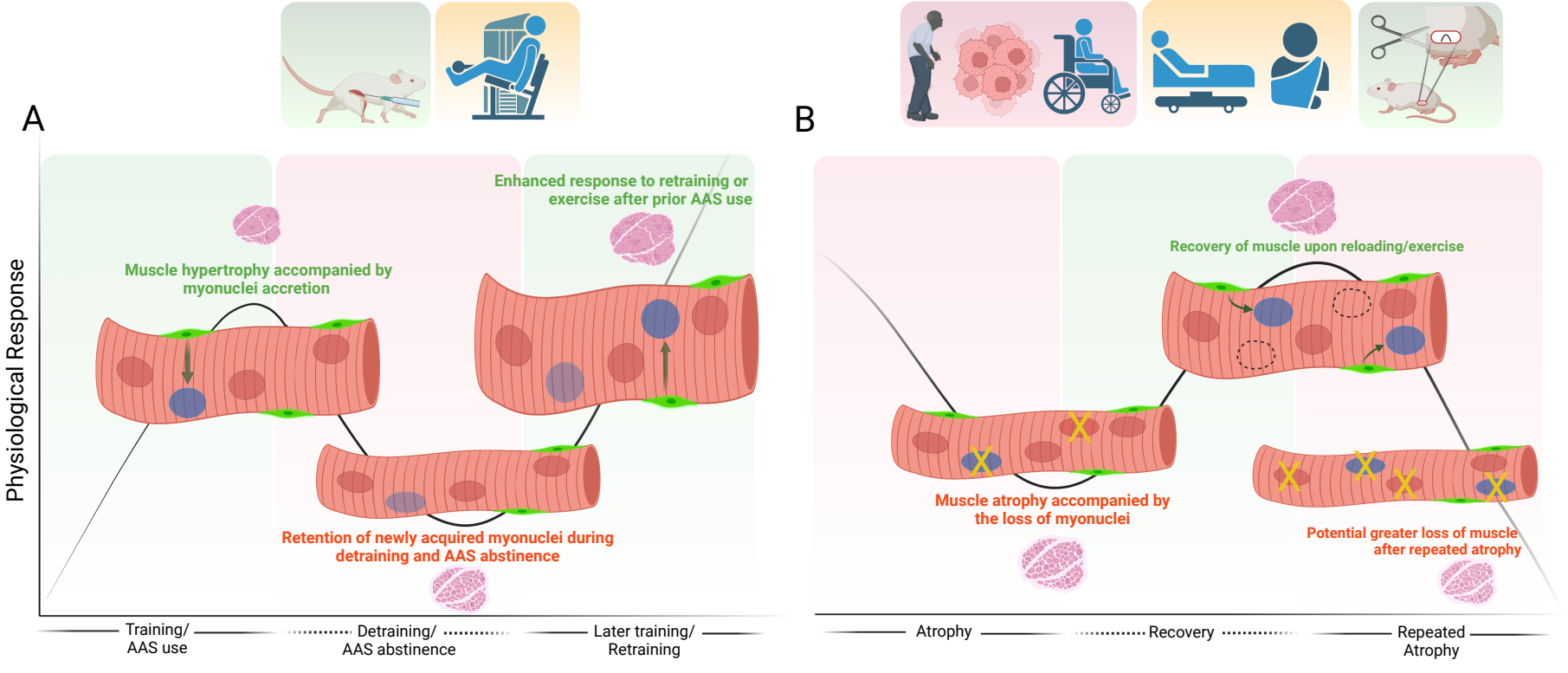
SJ, Fry CS, and Murach KA. A molecular signature defining exercise adaptation with ageing and in vivo partial reprogramming in skeletal muscle. *The Journal of Physiology* 601: 763-782, 2023.

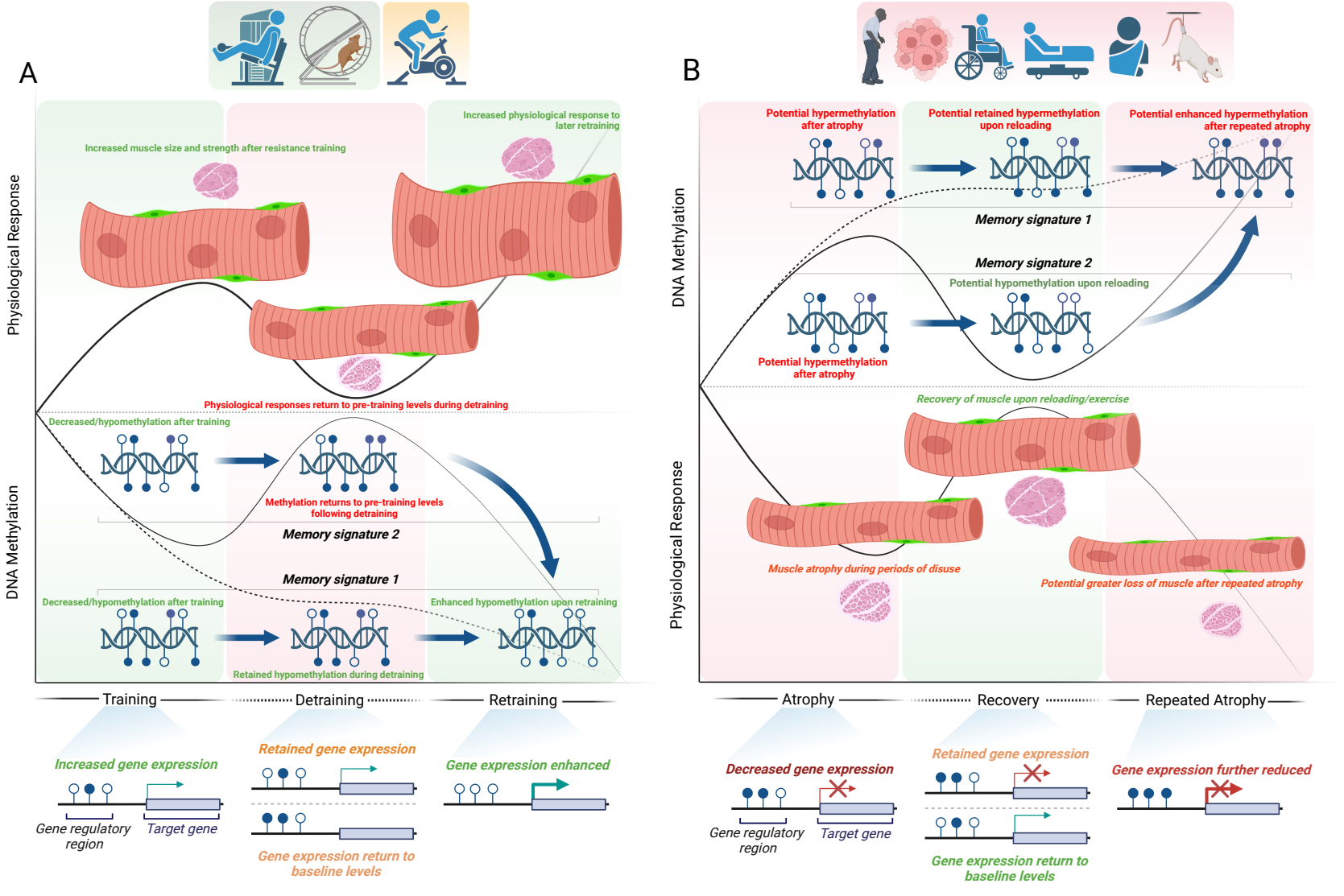
Figure Legends

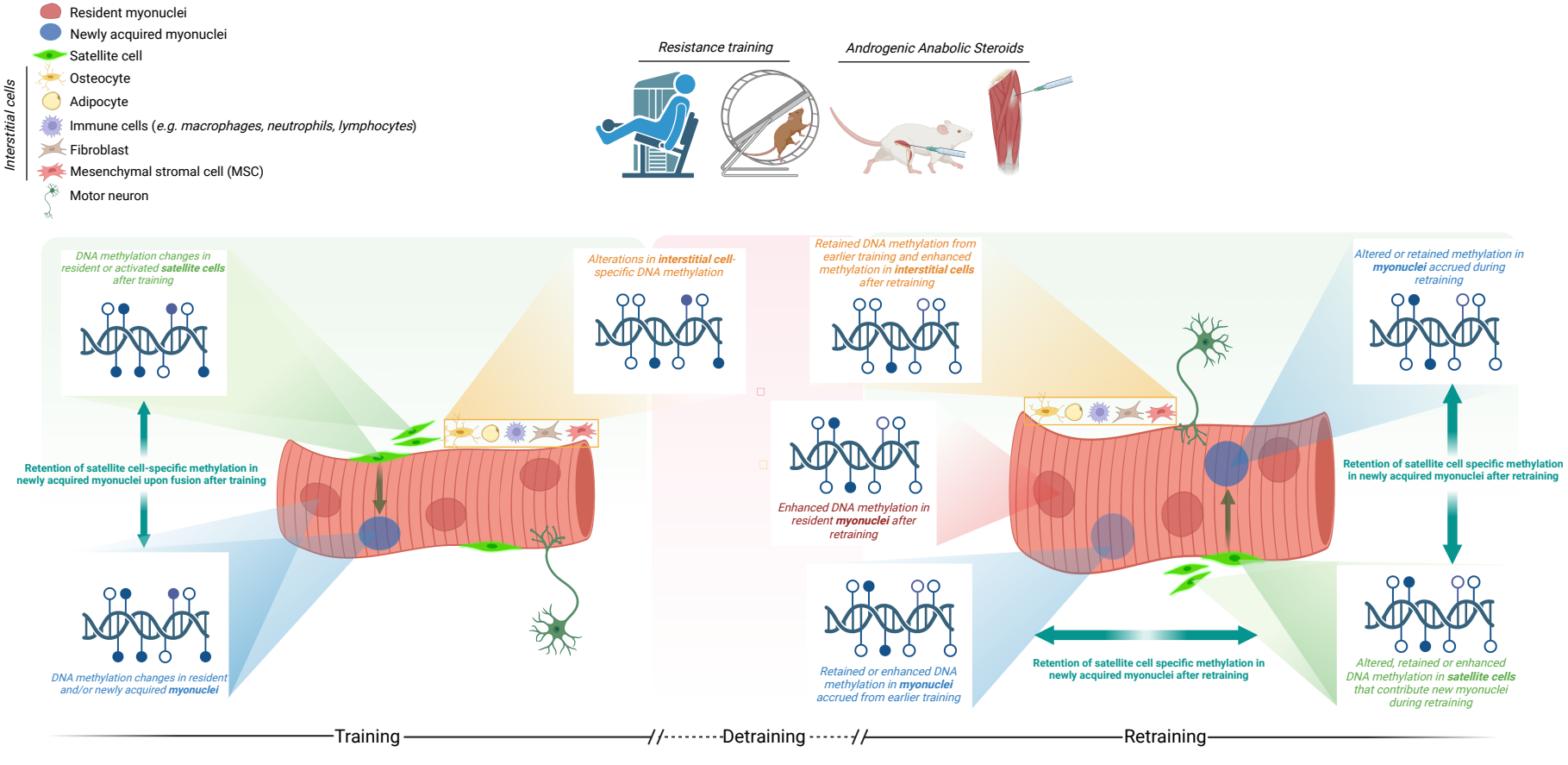
Figure 1. Proposed mechanisms for cellular muscle memory in response to **(A)** positive and **(B)** negative stimuli in skeletal muscle. **(A)** Androgenic anabolic steroid (AAS)- and/or resistance training (RT)-induced hypertrophy is accompanied by the fusion of satellite cells, contributing new myonuclei. In rodents newly acquired myonuclei are then retained during periods of muscle loss following detraining and/or AAS abstinence. Finally, the accrual and retention of new myonuclei after initial training and/or AAS use coupled with the potential acquisition of more myonuclei after retraining may explain the enhanced muscle response to later training. In human muscle this response is still to be fully confirmed. **(B)** Muscle loss during disuse atrophy (e.g., bed rest and/or immobilisation), aging and muscle-wasting diseases (e.g., type 2 diabetes and cancer cachexia) may be due to a loss of resident and/or newly acquired myonuclei. Upon reloading/exercise, the acquisition of new myonuclei may permit sufficient recovery of muscle. A potential greater loss of skeletal muscle following repeated atrophy could be due to a further loss of resident and/or newly acquired myonuclei. Loss of myonuclei with atrophy is still widely debated and therefore figure B is still largely hypothetical. Figure created with BioRender.com

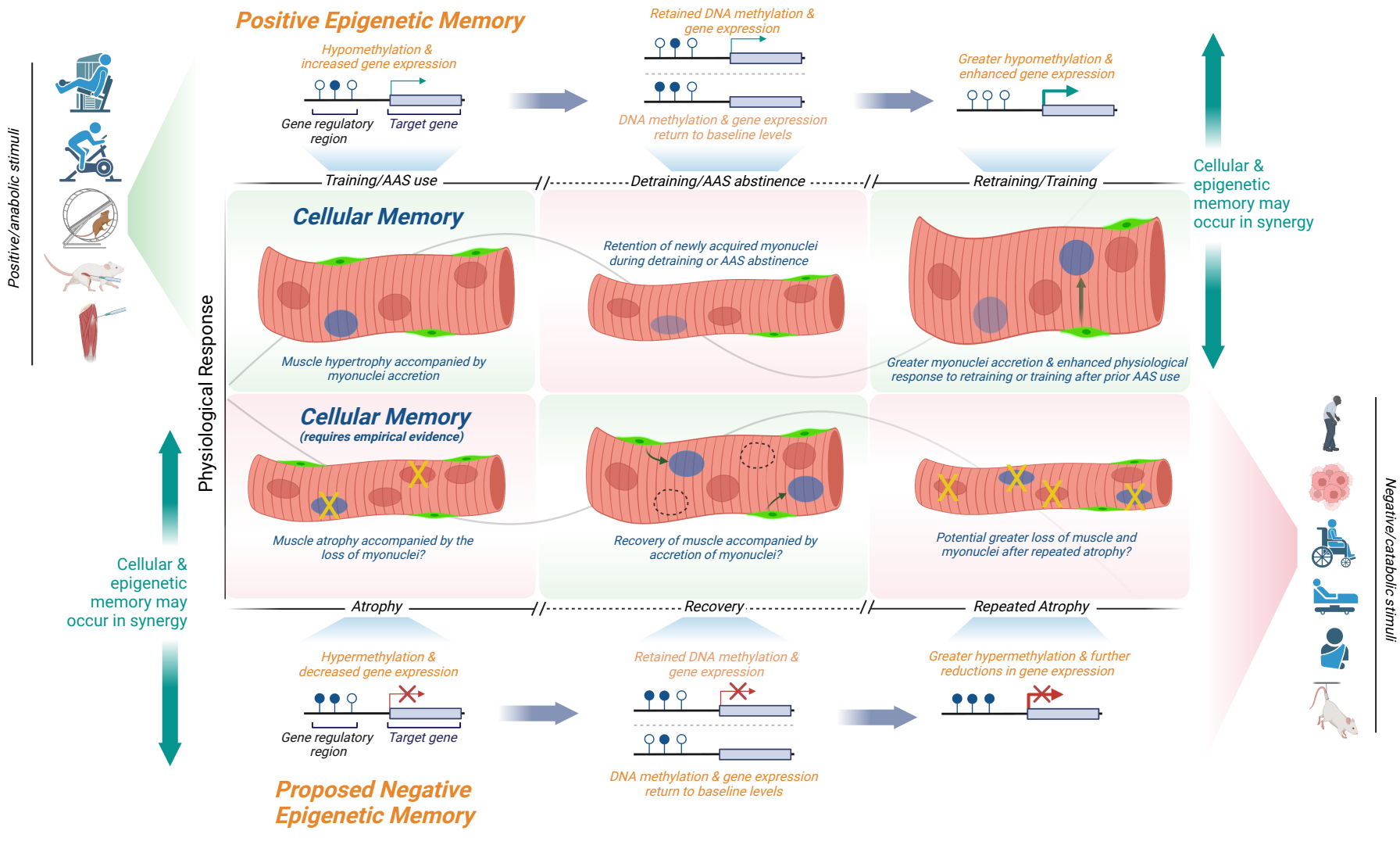
Figure 2. Proposed mechanisms for a **(A)** positive and **(B)** negative epigenetic memory of exercise in skeletal muscle. **(A)** The physiological response to resistance (e.g., increased muscle size) is accompanied by reduced methylation (i.e., hypomethylation) enabling gene transcription. DNA hypomethylation after training is then either retained into detraining (memory signature 1) and in some cases gene expression also remains elevated. Alternatively, methylation returns to baseline levels (memory signature 2) after detraining where at the physiological level, muscle returns to its´ pre-exercise training size. Finally, the enhanced response to resistance (e.g., muscle size and strength) exercise after later retraining is coupled with retained (memory signature 1) and in some instances even greater hypomethylation and enhanced gene transcription (memory signatures 1 and 2). **(B)** Oppositely, muscle loss in response to aging, muscle-wasting diseases, and disuse atrophy (e.g., bed rest or immobilisation) may result in increased DNA methylation (i.e., hypermethylation) and reductions in gene transcription. Upon reloading and recovery of muscle, DNA methylation levels may either be retained (memory signature 1) or return to baseline levels (depicted in memory signature 2). Finally, a potential greater loss of muscle mass and function after repeated disuse atrophy may be accompanied by enhanced hypermethylation (memory signatures 1 and 2) and perhaps even greater reductions in gene expression, suggestive of negative epigenetic memory of skeletal muscle wasting. This negative epigenetic memory hypothesis is not yet confirmed in empirical studies and requires future investigation. Open (clear) circles represent hypomethylation and closed (dark) circles represent hypermethylation. Figure created with BioRender.com

Figure 3. Proposed hypothetical mechanisms for the integration of both cellular and epigenetic memory mechanisms. Muscle (e.g., satellite cells and myonuclei) and non-muscle cells (e.g., interstitial cells including; osteocytes, fibroblasts, adipocytes, immune cells) resident in skeletal muscle tissue may demonstrate distinct DNA methylation changes in response to anabolic stimuli such as resistance exercise training. Distinct epigenetic profiles may also exist within the same cell type depending on its current state. For example, altered DNA methylation across resident, activated, and fusing satellite cells together with resident versus newly acquired myonuclei as a result satellite cell fusion may all demonstrate divergent epigenetic profiles in genes that are responsible for different functions. For example, growth-related epigenetic memory genes from myonuclei versus transcription factor, cell-cell signalling and ribosomal protein genes from satellite cell nuclei. Such exercise training-induced alterations in DNA methylation across different cell types may either be retained from earlier training or further enhanced when re-exposed to the same or similar stimuli in future. Such enhanced epigenetic changes are potentially coupled with greater myonuclei accretion. Epigenetic changes in satellite cells may also mean that future generations of cells can pass on these epigenetic changes to the fibre when incorporated as myonuclei during training and retraining. Figure created with BioRender.com


A
B
Physiological Response
Muscle hypertrophy accompanied by myonuclei accretion
Enhanced response to retraining or exercise after prior AAS use
Retention of newly acquired myonuclei during detraining and AAS abstinence
Training/ AAS use
Detraining/ AAS abstinence
Later training/ Retraining
Recovery of muscle upon reloading/exercise
Muscle atrophy accompanied by the loss of myonuclei
Potential greater loss of muscle after repeated atrophy
Atrophy
Recovery
Repeated Atrophy
Resident myonuclei
Satellite cell
Newly acquired (bold) and retained (shaded) myonuclei
Myonuclei that are acquired from previous exercise or during recovery from atrophy
Myonuclei that are lost during atrophy


A
Physiological Response
DNA Methylation
Increased muscle size and strength after resistance training
Increased physiological response to later retraining
Physiological responses return to pre-training levels during detraining
Decreased/hypomethylation after training
Methylation returns to pre-training levels following detraining
Memory signature 2
Memory signature 1
Decreased/hypomethylation after training
Enhanced hypomethylation upon retraining
Retained hypomethylation during detraining
Training
Detraining
Retraining
Increased gene expression
Gene regulatory region
Target gene
Retained gene expression
Gene expression return to baseline levels
Gene expression enhanced
B
DNA Methylation
Physiological Response
Potential hypermethylation after atrophy
Potential retained hypermethylation upon reloading
Potential enhanced hypermethylation after repeated atrophy
Memory signature 1
Memory signature 2
Potential hypomethylation upon reloading
Potential hypermethylation after atrophy
Recovery of muscle upon reloading/exercise
Muscle atrophy during periods of disuse
Potential greater loss of muscle after repeated atrophy
Atrophy
Recovery
Repeated Atrophy
Decreased gene expression
Gene regulatory region
Target gene
Retained gene expression
Gene expression return to baseline levels
Gene expression further reduced


Resident myonuclei
Newly acquired myonuclei
Satellite cell
Interstitial cells
Osteocyte
Adipocyte
Immune cells (e.g. macrophages, neutrophils, lymphocytes)
Fibroblast
Mesenchymal stromal cell (MSC)
Motor neuron
Resistance training
Androgenic Anabolic Steroids
DNA methylation changes in resident or activated satellite cells after training
Alterations in interstitial cell-specific DNA methylation
Retained DNA methylation from earlier training and enhanced methylation in interstitial cells after retraining
Altered or retained methylation in myonuclei accrued during retraining
Retention of satellite cell-specific methylation in newly acquired myonuclei upon fusion after training
Enhanced DNA methylation in resident myonuclei after retraining
Retention of satellite cell specific methylation in newly acquired myonuclei after retraining
DNA methylation changes in resident and/or newly acquired myonuclei
Retained or enhanced DNA methylation in myonuclei accrued from earlier training
Retention of satellite cell specific methylation in newly acquired myonuclei after retraining
Altered, retained or enhanced DNA methylation in satellite cells that contribute new myonuclei during retraining
Training
Detraining
Retraining


Positive/anabolic stimuli
Positive Epigenetic Memory
Hypomethylation & increased gene expression
Gene regulatory region
Target gene
Retained DNA methylation & gene expression
DNA methylation & gene expression return to baseline levels
Greater hypomethylation & enhanced gene expression
Training/AAS use
Detraining/AAS abstinence
Retraining/Training
Cellular Memory
Muscle hypertrophy accompanied by myonuclei accretion
Retention of newly acquired myonuclei during detraining or AAS abstinence
Greater myonuclei accretion & enhanced physiological response to retraining or training after prior AAS use
Cellular & epigenetic memory may occur in synergy
Physiological Response
Cellular Memory
(requires empirical evidence)
Muscle atrophy accompanied by the loss of myonuclei?
Recovery of muscle accompanied by accretion of myonuclei?
Potential greater loss of muscle and myonuclei after repeated atrophy?
Cellular & epigenetic memory may occur in synergy
Atrophy
Recovery
Repeated Atrophy
Hypermethylation & decreased gene expression
Gene regulatory region
Target gene
Retained DNA methylation & gene expression
DNA methylation & gene expression return to baseline levels
Greater hypermethylation & further reductions in gene expression
Proposed Negative Epigenetic Memory
Negative/catabolic stimuli