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Immunohistochemical Detection of Activating Transcription Factor 3, a Hub of the Cellular Adaptive–Response Network

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Abstract

Activating transcription factor 3 (ATF3) gene encodes a member of the ATF family of transcription factors and is induced by various stress signals, including many of those that induce the unfolded protein response (UPR). Emerging evidence suggests that ATF3 is a hub of the cellular adaptive–response network and studies using various mouse models indicate that ATF3 plays a role in the pathogenesis of various diseases. One way to investigate the potential relevance of ATF3 to human diseases is to determine its expression in patient samples and test whether it correlates with disease progression or clinical outcomes. Due to the scarcity and preciousness of patient samples, methods that can detect ATF3 on archival tissue sections would greatly facilitate this research. In this chapter, we briefly review the roles of ATF3 in cellular adaptive–response and UPR, and then describe the detailed steps and tips that we developed based on general immunohistochemistry (IHC) protocols to detect ATF3 on paraffin embedded sections.

1. Introduction

1.1. ATF3 as a hub of the cellular adaptive–response network

ATF3 is a member of the activating transcription factor/cAMP responsive element binding (CREB) protein family of transcription factors, which share the basic region-leucine zipper (bZip) DNA binding motif and bind to the consensus sequence TGACGTCA *in vitro* (for a previous review, see Hai and Hartman, 2001). The level of ATF3 mRNA is low or undetectable in normal unstressed tissues (from mice) and most cell lines, but greatly increases upon stimulation (Hai, 2006; Hai *et al.*, 1999). One striking feature of ATF3 induction is that it is neither tissue-specific nor stimulus-specific. ATF3 can be induced by a broad spectrum of stimuli and can be induced in various tissues or cell types. In fact, it is more an exception than the norm, if a stimulus does not induce ATF3. For a list of stimuli that have been shown to induce ATF3, see a previous review (Hai, 2006). Not surprisingly, many signaling pathways have been demonstrated to be involved in the induction of ATF3 by stress signals, including the JNK, p38, NFκB, PKC, and calcium signaling pathways (Hai *et al.*, 2010). Consistently, the ATF3 promoter is packed with transcription factor binding sites, many of which are recognized by factors downstream of the signaling pathways described above. Examples include ATF, Fos/Jun, NFκB (see Hai *et al.*, 2010 for a review), and NFAT (Wu *et al.*, 2010). In addition to the inducibility of ATF3 expression, amino acid sequence analyses of ATF3 revealed many potential sites for modification; for example, the presence of 21 serines/threonines, one tyrosine, and 17 lysines, which together account for ~20% of the molecule. Although rigorous evidence for posttranslational modifications of ATF3 and their functional significance is lacking at present, this abundance of potential

posttranslational modification sites is consistent with the idea that ATF3 is a target for various regulations. Taken together, ATF3 gene is induced by a variety of stress signals and signaling pathways, and its protein is a potential target for various modifications. All these indicate that ATF3 acts as an integration point for a variety of cellular controls, prompting us to put forth the idea that ATF3 functions as a “hub” of the cellular adaptive-response network that is utilized in response to the disturbance of homeostasis (more in a previous review, Hai *et al.*, 2010).

1.2. ATF3 in unfolded protein response

Endoplasmic reticulum (ER) stress refers to the condition whereby unfolded proteins accumulate in the lumen of ER. Under this condition, the cells activate a set of responses collectively called unfolded protein response (UPR), which is composed of three signaling arms distinguished by the following ER-membrane proteins that sense the unfolded proteins in the lumen: (a) IRE1, (b) ATF6, and (c) PERK (reviews: Kaufman, 1999; Ron and Walter, 2007; Schroder and Kaufman, 2005). ATF3 is induced by the PERK pathway and plays an integral role in coordinating this pathway's function (Jiang *et al.*, 2004). Upon activation, PERK phosphorylates eIF2 α and results in the increased production of ATF4 protein, which in turn upregulates ATF3 expression (Jiang *et al.*, 2004, and see Chapter 19). One interesting aspect of the PERK arm of UPR is that phosphorylation of eIF2 α can also be achieved by other eIF2 α kinases: GCN2, PKR, and HRI (for a review, see Wek *et al.*, 2006). These kinases can be induced by many stress signals unrelated to ER stress, such as nutritional stress, double stranded RNA accumulation, UV irradiation, and heat shock (reviews, Ron and Walter, 2007; Wek *et al.*, 2006). Thus, Ron and colleagues suggested referring to events downstream of eIF2 α phosphorylation as integrated stress response (ISR) (Harding *et al.*, 2003). Not surprisingly, all the stress signals that induce ISR also induce ATF3, presumably at least partly via the eIF2 α $\rightarrow$ ATF4 pathway. However, it is possible that they also induce ATF3 expression via other signaling pathways, such as the JNK, p38, and NF κ B pathways, since these pathways are known to be induced by the above stress signals and are known to be involved in the induction of ATF3. Taken together, ATF3 is induced by a variety of stress signals—including ER stress, stresses that induce ISR, and other stress signals (Fig. 11.1), again supporting the notion that ATF3 is a hub or integration point for cellular stress responses.

1.3. ATF3 in disease models

A survey of the literature indicates that ATF3 is implicated in a diversity of diseases based on various mouse models which employed either loss- or gain-of-function approaches (for a list, see Table 11.1). One general statement summarizes these studies: ATF3 has pleiotropic effects and the consequences of its expression can be beneficial or detrimental in a context-dependent manner. As an example, ATF3 inhibits the expression of pro-inflammatory genes, such as TNF α , IL12b, and IFN γ , in immune cells (Filen *et al.*, 2010; Gilchrist *et al.*, 2006; Rosenberger *et al.*, 2008; Whitmore *et al.*, 2007). In doing so, ATF3 prevents the immune system from overreacting and thus protects the whole organism in inflammatory disease models such as septic shock, asthma, and ventilation-induced lung injury (Akram *et al.*, 2010; Gilchrist *et al.*, 2006, 2008). However, the price for this immune dampening effect is that it weakens the host defense system, making it more vulnerable to infection, such as by cytomegalovirus (Rosenberger *et al.*, 2008). This underscores the double-edge sword nature of ATF3. Another example of context dependency is the role of ATF3 in cancer development. ATF3 was demonstrated to be oncogenic in several cancer models, such as breast cancer, prostate cancer, and skin cancer (Bandyopadhyay *et al.*, 2006; Wang *et al.*, 2008; Wu *et al.*, 2010; Yin *et al.*, 2010). However, it was also demonstrated to be anti-oncogenic in a colon cancer model and a Ras-mediated transformation model (Bottone *et al.*, 2005; Lu *et al.*, 2006). Although the anti-oncogenic results are paradoxical to the above

findings that ATF3 is oncogenic, they are consistent with many reports that ATF3 is pro-apoptotic and is deleterious to the tissues in transgenic mice that express ATF3 (for references, see Table 11.1 and a previous review Hai *et al.*, 2010). One potential explanation for these seemingly conflicting data is the degree of cell malignancy: ATF3 is pro-apoptotic to normal or untransformed cells, but protects the cells from death upon their malignant transformation as shown by an *in vitro* study using isogenic cell lines with varying degrees of malignancy (Yin *et al.*, 2008). However, much more work is required to understand the context-dependency of ATF3. For more description of ATF3 in different disease models, see previous reviews (Hai *et al.*, 2010; McConoughey *et al.*, 2010).

The above studies using mouse models raised an important question: does ATF3 play a role in human diseases? One approach to address this question is to examine ATF3 expression in human samples and ask whether it correlates with any disease states or clinical outcomes. Due to the scarcity and preciousness of patient samples, methods that can detect ATF3 on archival tissue sections would greatly facilitate the research. Immunohistochemistry (IHC) is a desirable assay for this purpose, because it detects proteins—the final products for many genes—and can reveal the subcellular localization of the molecules. In addition, it is generally compatible with paraffin embedded sections. Below, we describe a protocol that we have developed over the years by combining and modifying different IHC protocols.

2. An IHC Protocol for ATF3

IHC is a finicky technique with many idiosyncratic issues that cannot be solved in a predictable manner. For each protein, it is necessary to develop a protocol that is specifically tailored for it; sometimes even with many trials and errors, it may not be possible to work out a condition that yields the most desirable quality of images. For a comprehensive review on technical aspects of IHC, see (Ramos-Vara, 2005). In Section 2.3, we detail a protocol that worked for detecting ATF3 in various tissues; however, it may need further modification for specific applications. This is because many factors affect IHC outcomes, including the batch of antibodies, the abundance of the protein of interest in the samples, and the variation in fixation time and methods. We found that even when assayed under exactly the same conditions carried out side-by-side, different tissues fixed by the same facility may yield images of different quality. Thus, it is important to carry out pilot experiments using the same tissues as desired for the real experiment. For precious samples such as tissue microarrays (TMAs), pilot experiments using TMAs may not be feasible. Obtain a few paraffin blocks of individual samples of the same tissue types and work out the conditions. If “test TMAs” (with small number of tissue cores) are available, try out the conditions on them first.

2.1. Important factors for consideration in pilot experiments

Below are several key factors that affect IHC results and should be optimized in pilot experiments.

2.1.1. Primary antibodies—Several anti-ATF3 antibodies are commercially available. We have only compared the antibodies from Atlas (# HPA001562, against amino acids 1–113) and Santa Cruz (Clone C-19, #sc-188, against the C' terminal amino acids 163–181). They are both polyclonal antibodies generated in rabbits, but differ in their performance. In general, the Atlas antibody gives rise to better signal-to-background ratios and cleaner images. Figure 11.2 shows an example. In addition, the Atlas antibody is more consistent than the Santa Cruz antibody, providing more reproducible IHC results on both human and murine tissues. In our hands, it is also more consistent in Western blot. Some batches of the Santa Cruz antibody detect nonspecific bands, which may account for its high background (darker and less specific signals compared to Atlas) and inconsistency in IHC. Due to its

better performance, we recommend the Atlas antibody for IHC, despite its higher cost. For each batch of antibody, it is important to titrate the antibody for the intended application. A good starting point is 1:50, 1:125, 1:150, 1:200, and 1:300. We found that dilution in a surprisingly narrow range (such as 1:125 vs. 1:150) could make a difference in the signal-to-background ratio. After optimization, set aside the current batch of antibody for IHC use only and purchase more of the same batch (if desired). Considering the importance of primary antibodies in IHC, it is possible that the development of new antibodies recognizing different epitopes within ATF3 may further improve this assay.

2.1.2. Antigen retrieval—Fixation cross-links and modifies the protein structure, reducing the recognition of epitopes by the antibodies (Ramos-Vara, 2005). To counter this problem, various antigen retrieval methods have been developed to reverse at least some, if not all, of the cross-linking (for a review, see Ramos-Vara, 2005). Many of the retrieval methods involve a heating step, but the means of heating vary; for example, pressure cooker, steamer, microwave oven, and hot plate. In our hands, the pressure cooker method worked the best for detecting ATF3 by IHC.

2.1.3. Secondary antibody and the detection methods—The commonly used biotin–avidin system for detecting IHC signals can produce high background, especially when harsh antigen retrieval methods are used (a review, Ramos-Vara, 2005). A polymer-based system independent of avidin–biotin was developed in the 1990s (see Sabattini *et al.*, 1998 and references therein). It consists of an inert polymer backbone conjugated with secondary antibodies and many (up to 100) molecules of reporter enzyme, such as peroxidase. Figure 11.3 shows a schematic diagram of two commercially available polymer-based systems: (a) ImmPRESS™, which uses a flexible micropolymer as the backbone, and (b) EnVision™, which uses dextran as the backbone. The polymer-based system offers two main advantages over the biotin–avidin system: first, since it is a two-step method, it is rapid and simple compared to the three-step biotin–avidin system; second, it has no background staining due to endogenous biotin or avidin (Ramos-Vara and Miller, 2006). A side-by-side comparison indicated that ImmPRESS™ performed better than EnVision™ in the majority of cases examined in the study (Ramos-Vara and Miller, 2006). We have used the ImmPRESS™ system and found it greatly improved the IHC signals for ATF3. However, we have not compared ImmPRESS™ to EnVision™ side-by-side.

Many different peroxidase substrates are available for the colorimetric development of the stained tissues. We routinely use two different peroxidase substrates depending on the application: (a) DAB, which gives rise to a brown color and is slower to develop, and (b) ImmPACT NovaRed, which gives rise to a red color and is more rapid to develop. Both substrates provide excellent color contrast when counterstaining with hematoxylin; however, there are unique advantages and disadvantages of each substrate that will dictate when their use is desirable. The slower development of the DAB substrate allows for the ability to more readily discern different signal intensities within the same tissue, possibly due to tighter control of the time required to obtain the desired signal intensity. However, we have observed that signals produced by staining endogenous ATF3 using the DAB substrate are generally less sharp than those produced with the ImmPACT NovaRed substrate. Regardless of the detection method used, one needs to carry out a pilot experiment for each project in order to determine the peroxidase reaction time for optimal staining. It may be necessary to try several time points and repeat the pilot experiments a few times to find the appropriate reaction time. See Section 2.3.5 for detailed steps.

2.1.4. Signal specificity—As with any assay, it is imperative to test the specificity of the signals. Here, we present our data on ATF3 using human breast cancer samples with the following tests. (a) Depletion: We carried out depletion experiments to test the specificity.

As shown in Fig. 11.4, pre-incubation of the antibody with the GST-ATF3 fusion protein completely removed the signals but pre-incubation with the same concentration of GST did not. (b) Control samples: As described in Section 1.1, ATF3 level is low or undetectable in unstressed mouse tissues. We used the breast reduction samples as a potential unstressed control tissue. In side-by-side experiments, the signal for ATF3 was greatly reduced in the breast reduction samples compared to that in the breast cancer samples (Fig. 11.5). However, this was not absolute. In most cases we could still detect a low level of ATF3, and in some cases the signal of ATF3 was quite strong (data not shown). These results are similar to that reported by MacLeod and colleagues, who analyzed ATF3 in human breast samples (Wang *et al.*, 2008). We posit that the breast reduction samples are not un-stressed tissues, a notion supported by the abundance of fibrotic material in their stroma. (c) Tumor samples from ATF3-deficient mice: Due to the unavailability of truly unstressed human tissues as ATF3-negative controls, we resorted to ATF3 knockout (KO) mice. We crossed the ATF3 KO mice to transgenic mice expressing the polyoma middle T (PyMT) antigen under the control of the murine mammary tumors virus (MMTV) promoter—MMTV-PyMT mice (Davie *et al.*, 2007; Guy *et al.*, 1992)—to generate tumor-bearing mice in the ATF3 KO background (C57BL/6-aft3^{-/-}-Tg(MMTV:PyMT)). We then analyzed tumors from these mice in parallel with that from the wild type counterparts (C57BL/6-aft3^{+/+}-Tg(MMTV:PyMT)). As shown in Fig. 11.6, the WT tumors contained many ATF3-positive cells but the KO tumor did not, supporting the specificity of the IHC stain. We note that the optimal dilution of the primary antibody for mouse tumors may be different from that for human tumors and should be determined empirically. Two cautionary notes are relevant here. (i) Although immunoglobulin G (IgG) is a commonly used negative control for immunological assays, we found it difficult to use in this case. As described above, the ATF3 signal-to-background ratio is affected by the dilution of the primary antibody—within a surprisingly narrow range. Since the purity of the control IgG and anti-ATF3 IgG may not be the same in the commercially available preparations, it is difficult to make a fair comparison. Even at the same final protein concentration, their effective concentrations may not be the same. In experiments where a slight change in dilution makes a big difference, this is a significant issue. In addition, the subclasses (IgG₁₋₄) and storage (longer storage results in aggregation) of IgG affect their ability to cause background signals (see Ramos-Vara, 2005 for a review), further diminishing the utility of IgG as controls. (ii) As described in Section 1.1, the induction of ATF3 is neither tissue-specific nor stimulus-specific. Thus, in a given specimen from diseased tissues, ATF3 may be present in more than one cell type. The broad expression of ATF3 *per se* should not be interpreted as nonspecificity. Controls should be carried out for proper data interpretation, and importantly—as with all experiments—the conclusions should be tested by different assays and approaches.

2.2. Required materials

Samples

- Human breast carcinoma, formalin-fixed paraffin embedded (FFPE) sections (4–5 μ m)
- Human breast reduction samples, FFPE sections (4–5 μ m)
- Mouse mammary tumors derived from WT mice expressing the MMTV-PyMT transgene, FFPE sections (4–5 μ m)
- Mouse mammary tumors derived from ATF3 KO mice expressing the MMTV-PyMT transgene, FFPE sections (4–5 μ m)

Devices

- Pressure cooker, 4 quart (Mantra Inc., #34111)

- Tissue Tek slide staining set: 12 staining dishes, rack which holds 12 staining dishes, 24-slide staining holder (Cardinal Health, #S7626-12)
- Glass baking dish (9" × 13")
- Metal slide staining tray (McCrone Microscopes and Accessories, #273-TRAY)
- ImmEdge hydrophobic barrier PAP pen (Vector Laboratories, #H-4000)
- Slides (Fisher Scientific, #12-550-15)
- Cover slips (Fisher Scientific, #12-548-56)
- Air vacuum device for gentle suction

Reagents and buffers

- Anti-ATF3 antibody from Atlas (# HPA001562): Polyclonal antibodies against ATF3 amino acids 1–113, made in rabbits
- Xylene (Histological grade, Fisher Scientific, #X3P-1GAL)
- Ethanol (200 proof, Fisher Scientific, BP2818-4)
- Deionized water (dH₂O)
- Hydrogen peroxide (Sigma, #H1009-500ML)
- *Wash buffer*: 1× Tris buffered saline with Tween-20 (TBST): 150 mM sodium chloride, 100 mM Tris–HCl, 0.1% Tween-20. Adjust pH to 7.5 before adding Tween-20.
- *Antigen unmasking solution (freshly prepared)*: 10 mM sodium citrate buffer, 0.05% Tween-20. Adjust pH to 6.0, before adding Tween-20.
- *Blocking solution*: 5% normal goat serum diluted in 1× wash buffer
- *ImmPRESSTM Polymer-based detection reagent*: anti-Rabbit IgG conjugated with peroxidase (Vector, #MP-7401)
- *Substrate for peroxidase*: (a) ImmPACT NovaRed dark red peroxidase substrate (Vector, #SK-4805) or (b) DAB substrate kit for peroxidase (Vector, #SK-4100)
- Hematoxylin (Fisher, #22-220-102)
- VectaMount mounting medium (Vector, #H-5000)

Disposables

- Gloves
- Paper towels
- Kimwipes
- Plastic wrap

2.3. Procedures

For all steps below after deparaffinization, do not allow slides to dry. For all wash steps and some incubation steps below, use the slide holder, which holds multiple slides standing-up. Put it in a plastic slide staining dish filled with ~250 ml of solution to make sure that the slides are entirely immersed (Fig. 11.7A). Although the slide holder can accommodate 24 slides, use it for maximal 12 slides with an empty slot between the adjacent slides. This prevents accidental contact between the slides. For the steps that do not use the slide holder,

such as incubation with the blocking solution, primary antibody, secondary antibodies, or staining solution, the slides should be laid flat on a staining tray with the tissue-side up and placed in a moist chamber as detailed below.

2.3.1. Deparaffinization and rehydration

- Incubate the slides in a slide holder at 60 °C in an oven for 1 h to melt the paraffin. Put the slide holder in an empty slide staining dish, which keeps the slide holder steady. After this step, do not allow the slides to dry out throughout the entire protocol.
- Incubate the slides in xylene for 5–10 min; move the slides to a new container with fresh xylene for another 5–10 min.
- Incubate the slides in 100% ethanol for 5 min, move the slides to a new container with fresh 100% ethanol for a second 5 min incubation.
- Incubate the slides in 95% ethanol for 5 min, move the slides to a new container with fresh 95% ethanol for a second 5 min incubation.
- Incubate the slides one time in 75% ethanol for 5 min.
- Incubate the slides in dH₂O for 5 min, move the slides to a new container with fresh dH₂O for a second 5 min incubation.

2.3.2. Antigen retrieval using the pressure cooker method

- While hydrating the tissue sections, prepare 2 l of antigen unmasking solution and heat it in a pressure cooker until the buffer starts to boil. Do not seal the lid, since you do not want the pressure to build yet. This takes approximately 40 min, about the same time that it takes to hydrate the tissues.
- After hydration, transfer the slides to the boiling buffer. Lay the slide holder on its side as illustrated in Fig. 11.7B, so that it does not accidentally fall during incubation. Seal the lid of the pressure cooker and allow the pressure to build to its maximum (this takes about 4 min once the lid is sealed and may vary depending on the pressure cooker).
- Keep the slides at this pressure for 20 min before turning off the heat, gradually releasing the pressure, and carefully opening the lid of the pressure cooker (this takes less than 5 min and may vary depending on the pressure cooker).
- Cool the antigen unmasking solution by placing the open pressure cooker under slow-running tap water for 10 min, being careful not to dispense the water directly over top of the slides.

2.3.3. Blocking and incubation with the primary antibody

- Wash the slides in dH₂O for 5 min, three times.
- Incubate the slides in 3% hydrogen peroxide (diluted in methanol) for 10 min to block the endogenous peroxidase activity.
- Wash the slides in dH₂O for 5 min, twice.
- Incubate the slides in wash buffer one time for 5 min.
- Set up the incubation with the blocking solution by handling one slide at a time. This step should be carried out carefully but quickly to prevent the tissues from drying. Remove a slide from the buffer and damp dry it by holding it up-right with its bottom edge touching a paper towel as illustrated in Fig. 11.7C. The capillary

action will drain the buffer from the slide. Then, use gentle air vacuum to dry a circle around the tissue—at a distance so as to not disturb the tissue—as illustrated in Fig. 11.7C. Use the PAP pen to trace the dry circle; this will create a heat-stable water-repellent barrier surrounding the tissue sections and allow reagents to remain on top of the sections. Note that the PAP pen will not work if the surface is wet. Lay the slides down flat on a slide staining tray with the tissue-side up. Immediately, load 100–400 μ l of blocking solution over the tissue section (until the section is completely covered).

- After finishing all slides, put the tray in a moist chamber for incubation. To set up a moist chamber, put two layers of damp, but not dripping-wet, paper towels in the bottom of a 9" $\times$ 13" glass baking dish, which accommodates the slide staining tray. Cover the dish with plastic wrap.
- Incubate at room temperature for 1 h.
- Remove the blocking solution by gentle air vacuum suction. Make sure to remove residual solution to avoid affecting the concentration of the primary antibody that follows.
- Load 100–400 μ l of the anti-ATF3 Atlas primary antibody diluted in blocking solution to each PAP pen-enclosed area (until the section is completely covered). The optimal dilution varies for different batches of antibodies and should be determined empirically for each batch. Refer to Section 2.1.1.
- Set up a moist chamber as above and incubate the slides at 4 °C overnight.
- Remove the primary antibody, place the slides in a slide holder, and wash the slides in wash buffer for 5 min, three times.

2.3.4. Incubation with the ImmPRESS™ secondary antibody

- Remove the wash solution by gentle air vacuum suction. Make sure to remove residual solution to avoid affecting the concentration of the secondary antibody that follows.
- Load 100–400 μ l of undiluted anti-rabbit Ig ImmPRESS™ reagent (until the section is completely covered).
- Set up a moist chamber and incubate the slides at room temperature for 30 min.
- Wash the slides in wash buffer for 5 min, three times.

2.3.5. Staining by peroxidase reaction

- Remove the wash solution by gentle air vacuum suction. Make sure to remove residual solution to avoid affecting the concentration of the peroxidase substrate solution that follows.
- Load 100–400 μ l of undiluted peroxidase substrate solution until desired staining intensity develops. As indicated in Section 2.1.3, the reaction time should be determined beforehand by pilot experiments. However, due to potential experimental variations, close inspection should be made; it may be necessary to terminate the reaction slightly before or after the predetermined time for optimal color development. To maintain the substrate development time as close as possible among all slides, divide the samples in groups of five and carry out this step in a batch-wise manner. Add the substrate to slide 1 and note the time elapsed between adding solution to slide 1 and adding solution to slide 2. Do the same for the subsequent slides. Terminate the reaction in the same sequence with the same

elapsed time. We recommend two substrate solutions: ImmPACT NovaRed and DAB substrate kit for peroxidase (see Section 2.2, reagents). Each has its pros and cons. (i) ImmPACT NovaRed is a rapid stain and takes 2–5 min for color development, in our experience, using different tissue samples. Its color is vivid and provides a good contrast against the blue hematoxylin counter stain. However, because it develops fast, experimental variation may introduce sufficient artificial differences to affect data interpretation. (ii) DAB is a slower developing stain than ImmPACT NovaRed; in our experience the reaction time ranges from 8 to 12 min. Thus, it is less sensitive to experimental variation. However, its color is less vivid and the resulting image is not as sharp. We recommend using ImmPACT NovaRed when the quality of image is important, but using DAB when the relative signals between samples are critical.

- To terminate the reaction, immerse the slides in dH₂O. It is not necessary to remove the substrate solution by suction. Simply move the slides to the slide holder that is placed inside a slide container with ~250 ml dH₂O. Then, rinse the slides in tap water by changing the water in the container five times. Remove the slide holder from the container while refilling it with water to avoid dislodging the tissue sections from the slides.

2.3.6. Counterstain and coverslipping

- Counterstain with freshly diluted hematoxylin (in dH₂O). The intensity of the counterstain should be faint, so that it does not interfere with data interpretation. It is best to consult the pathologist(s) who will score the IHC signals to decide the desired intensity. The precise dilution and incubation time to achieve the desired counterstain should be determined empirically and can vary depending on the batch and age of hematoxylin. A good starting point is 1:10 dilution for 1 min. After counterstaining, observe the slides closely. As soon as the desired color contrast is present, immerse the slides in dH₂O.
- Dehydrate the slides by serial incubation in series of graded alcohols: 70% (2X) → 95% (2X) → 100% (2X), for 2 min each.
- Incubate the slides in xylene for 5–10 min to dissolve the hydrophobic edge drawn around the section with the PAP pen.
- Mount by carefully placing a small drop of VectaMount permanent mounting media on the tissue section and slowly laying a cover slip on top. Start by lowering the cover slip on one side of the section; the surface tension of the mounting media will draw the cover slip close. This technique usually avoids trapping air bubbles in between the cover slip and tissues. Using Kimwipes, carefully blot away excess mounting media from the edges of the cover slip. Allow mounting media to dry by laying slides flat and incubating at room temperature overnight. Note that since the mounting media is non-aqueous, it is not necessary to further seal around the edge of the cover slip.

2.3.7. Semi-quantification of the IHC signals—If comparing signal intensity is an important component of the project, it is essential to carry out IHC on all sides simultaneously—side-by-side. It is also essential to have experienced pathologists to score the signals. Ideally, the pathologists should be unaware of—and thus unbiased by—the hypothesis, and the IHC signals should be scored by more than one pathologist.

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Abbreviations

ATF3	activating transcription factor 3
CREB	cyclic AMP responsive element binding protein
bZip	basic region leucine zipper
ER	endoplasmic reticulum
eIF2	eukaryotic initiation factor 2
UPR	unfolded protein response
ISR	integrated stress response
HRI	heme regulated inhibitor kinase, also called eIF2 α kinase (EIF2AK1)
PKR	RNA-dependent protein kinase, also called EIF2AK2
PERK	PKR-like endoplasmic reticulum kinase, also called pancreatic eIF2 kinase (PEK), or EIF2AK3
GCN2	general control nonrepressible 2, also called EIF2AK4
IRE1	inositol-requiring enzyme 1
LPS	lipopolysaccharide
UV	ultraviolet
IHC	immunohistochemistry

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Figure 11.1.

A schematic of ATF3 induction by various stress signals. See text in Section 1.2 for details. ER, endoplasmic reticulum; eIF2, eukaryotic initiation factor 2; UPR, unfolded protein response; ISR, integrated stress response; HRI, heme regulated inhibitor kinase, also called eIF2alpha kinase (EIF2AK1); PKR, RNA-dependent protein kinase, also called EIF2AK2; PERK, PKR-like endoplasmic reticulum kinase, also called pancreatic eIF2 kinase (PEK), or EIF2AK3; GCN2, general control non-repressible 2, also called EIF2AK4; IRE1, inositol-requiring enzyme 1; LPS, lipopolysaccharide; UV, ultraviolet.

Figure 11.2.

An IHC comparison of the Atlas and Santa Cruz anti-ATF3 antibodies. Human breast tumor sections were stained using optimized conditions for Atlas (1:125 dilution; A) or Santa Cruz (1:200 dilution; B) anti-ATF3 antibodies. Staining was performed with ImmPRESS anti-rabbit secondary antibody, developed using ImmPACT NovaRed substrate, and counterstained with hematoxylin. Scale bars: 200 μ m.

Figure 11.3.
 A schematic of two commercially available polymer-based systems. A flexible micropolymer backbone (ImmPRESS reagent; A) or dextran (Envision++ reagent; B) bridges a large number of peroxidase molecules to each secondary antibody. Ab, antibody.

Figure 11.4.

Specificity test of the Atlas anti-ATF3 antibody. Atlas anti-ATF3 antibody solution was either untreated (A), or precleared using GST (B), or GST-ATF3 (C). The resulting solutions were used to stain human breast tumor sections. Conditions were the same as those described in the Fig. 11.2. Scale bars: 100 μ m.

Figure 11.5.

A comparison of ATF3 expression in human breast reduction (A) and breast tumor (B) samples. Tissue sections were stained using the same conditions described in the Fig. 11.2. Scale bars: 100 μ m.

Figure 11.6.

Analyses of ATF3 in tumors derived from WT or ATF3 KO mice. Serial sections of tumors from MMTV-PyMT transgenic mice in either WT (A, B) or ATF3 KO (C, D) background were stained with hematoxylin and eosin (A, C) or Atlas anti-ATF3 antibody (B, D). The slower developing DAB substrate was used in this experiment. No counterstain was applied, as it reduced the ability to discern different signal intensities. Scale bars: 100 μ m.

Figure 11.7.
Schematics of some devices and procedures described in Section 2.3.

Table 11.1

A list of mouse models implicating ATF3 in various diseases

	Models^a	Phenotypes	References
Cell types with ectopic expression of ATF3	<u>Transgenic mice</u>		
	Hepatocytes	Liver dysfunction	Allen-Jennings <i>et al.</i> (2001, 2002)
	Pancreatic β -cells	Reduced β -cell mass and defects in glucose homeostasis	Li <i>et al.</i> (2008)
	Cardiac myocytes	Conduction abnormalities and contractile dysfunction	Okamoto <i>et al.</i> (2001)
	Basal epithelial cells	Epidermal hyperplasia, oral carcinoma, and mammary carcinoma (in biparous mice)	Wang <i>et al.</i> (2007, 2008)
Stress models	<u>ATF3 KO mice^b</u>		
	LPS-induced Septic shock	Hyper-inflammation and animals succumb faster	Gilchrist <i>et al.</i> (2006)
	Pulmonary stress	Hyper-inflammation in both allergy and ventilator-induced lung injury stress models	Akram <i>et al.</i> (2010), Gilchrist <i>et al.</i> (2008)
	^c MCMV infection	Decreased viral load	Rosenberger <i>et al.</i> (2008)
	High fat diet	Decreased ability of β -cells to function and increased glucose intolerance	Zmuda <i>et al.</i> (2010)
Ectopic expression of ATF3 in cancer cells	<u>Injection models</u>		
	Prostate, keratinocyte, melanoma, and breast cancer cells	Enhanced tumorigenicity or metastasis	Bandyopadhyay <i>et al.</i> (2006), Ishiguro <i>et al.</i> (1996), Wu <i>et al.</i> (2010), Yin <i>et al.</i> (2010)
	Colon cancer cells	Reduced tumorigenicity	Bottone <i>et al.</i> (2005)

Phenotypes indicate the consequences of ATF3 deficiency in the corresponding stress models or the consequences of ectopically expressing ATF3 (transgenic or injection models).

^a Gain-of-function approach.

^b Loss-of-function approach.

^c ATF3 KO has a beneficial effect.