# ON THE CONNECTION BETWEEN THE TRANSVERSE TUBULES AND THE PLASMA MEMBRANE IN FROG SEMITENDINOSUS SKELETAL MUSCLE

## Are Caveolae the Mouths of the Transverse Tubule System?

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The transverse tubular system (TTS) of skeletal muscle fibers represents the morphological basis for the inward spread of conduction of the electrical signal that triggers muscle contraction. A historical account of the main steps contributing to the elucidation of the structure and function of the TTS has been presented by Huxley (1971).

While the localization of the TTS and its association with the sarcoplasmic reticulum (SR) is well documented, there is still a need further to develop our knowledge of the morphology of the connection between the TTS and the plasma membrane. It is generally believed that the TTS opens directly to the extracellular space and that there is continuity between its membrane and the sarcolemma. However, direct observation of such a connection has been clearly shown only for the myotome of fish (Franzini-Armstrong and Porter, 1964). In other muscle fibers, only indirect evidence of the connection has been provided by experiments showing penetration of extracellular tracers into the TTS. These extracellular markers were also observed inside another membrane-bounded compartment consisting of round profiles named "caveolae" (Yamada, 1955) or "pinocytotic vesicles" [1] (Ashurst, 1969).

The present study deals with the communication between the TTS, caveolae, and plasma membrane (Peachey, 1965; Ezerman and Ishikawa, 1967; Schiaffino and Margreth, 1968; and Rayns et al., 1968). A detailed study of the caveolae compartment was undertaken with ruthenium red as an electron-dense tracer. As a result of this study, we propose that in certain species the caveolae compartment represents the transitional region in the connection between the TTS and the sarcolemma.

[1] The term "pinocytotic vesicles" is inadequate for this membrane-bounded compartment, because in adult fibers the round profiles are permanently open to the outside and exclusively located under the sarcolemma.

## MATERIALS AND METHODS

Experiments were done on the semitendinosus muscle of the frog *Rana pipiens*. The muscles of both legs were removed and kept in frog Ringer's solution with 10 mg/liter of *d*-tubocurarine, until they were transferred to a chamber for dissection. A bundle of about four to eight fibers was dissected from the muscle at room temperature, stretched 1.2 times its slack length, and fixed.

Fixation was carried out in 3% glutaraldehyde-$H_2O_2$ in 0.1 M phosphate buffer, pH 7.4 (Peracchia and Mittler, 1972). Postfixation was done with 1% osmium tetroxide in 0.1 M sodium cacodylate buffer, pH 7.3. The samples were dehydrated in alcohol, passed through propylene oxide, and embedded in Epon 812.

Thin sections were cut with an LKB Ultramicrotome by use of a diamond knife. The sections were supported on 200 mesh carbon-coated grids and double stained with uranyl acetate and lead citrate. Observations were made with an AEI-6B electron microscope at 80 kV. Some grids were examined with a Philips EM 300 electron microscope equipped with a tilting and rotation stage. Tilting series (from $-36°$ to $+36°$) were performed.

Different concentrations of ruthenium red (100, 200, and 300 parts per million) were added to the osmium tetroxide solution at room temperature (Luft, 1971). Ruthenium red was used as an extracellular tracer because it heavily stains the basement lamina as well as the lumen of caveolae and transverse tubules.

In other experiments, after postfixation in osmium tetroxide, the samples were washed five times in 0.1 M sodium acetate (pH 5.5) and block stained with 1% uranyl acetate prepared in the same buffer. The samples were left in this solution overnight at room temperature.

## RESULTS

### *The Caveolae Compartment*

The term caveola defines a round or slightly flattened membrane-limited profile about 100 nm in diameter, located below the sarcolemma of the muscle fiber.

In logitudinal sections (Fig. 1) the caveolae are observed closely associated with the plasma mem-

brane and aligned in a single row. Each row is made up of a group of 7–15 adjacent caveolae located mainly at the I band, but also spreading over the other bands of the sarcomere. In transverse sections (Fig. 2) similar groups of caveolae are observed, along the perimeter of the fiber, at the I band. However, scattered caveolae are also present, as shown in Fig. 2 (small arrows).

As indicated by the presence of ruthenium red in the lumen of caveolae, the caveolae compartment communicated with the extracellular space. The extension of the communication can be inferred by noticing that at least 90% of the caveolae observed were filled with the tracer. A direct observation of such a communication is shown in Fig. 3. The plasma membrane and the caveolae membranes are continuous via a narrow channel about 17 nm in diameter by 25 nm in length.

In longitudinal and transverse views, the caveolae appear as "vesicles"; however, about 25% of adjacent caveolae counted in this study were continuous with other caveolae through narrow stalks. As an example of these connections, Fig. 4 (arrow) shows a profile, 240 nm in length and 90 nm in width, of approximately twice the length of a single caveola.

Besides their connection to the extracellular space and their fusion to adjacent caveolae, some caveolae are also continuous with a narrow, tortuous tubular profile oriented parallel to the fiber surface. Even though this tubule may represent another type of communication between caveolae, it is also possible that it is part of the T system of the fiber (Figs. 5, 6), free of its association with the SR.

### *Caveolae at the Myotendon Junction*

At the myotendon junction the muscle fiber is divided into numerous finger-shaped projections (Hanak and Bock, 1971). While maintaining the same characteristics with regard to shape, diameter, and connection to the extracellular space, the caveolae display a different arrangement within these projections. As shown in Fig. 7, fusion between adjacent caveolae and their continuity with deformed tubular elements are observed more often in this region (arrow, Fig. 7).

### *Distribution of the Caveolae with Respect to the Sarcomere*

The typical pattern of distribution of caveolae with respect to the bands of the sarcomere is seen in Figs. 1 and 2. In longitudinal sections, discrete groups of caveolae are centered at the I band, but also spread over the other bands of the sarcomere (Fig. 8). In transverse sections, discrete groups of caveolae were also found facing the I band. This pattern suggests a preferential distribution of caveolae at the I band. In order further to examine this possibility we performed an analysis of the density of caveolae at each band of the sarcomere.

The histogram in Fig. 8 shows that the largest density of caveolae is associated with the sarcolemma facing the I band. An estimate of the average density of caveolae over the fiber surface, as obtained from thin sections, is about $3 \times 10^9$ caveolae/cm$^2$. This value does not include the caveolae located at the myotendon junction. Although the calculated density of caveolae is only a rough approximation, the number of caveolae is clearly severalfold larger than the number of "sensitive spots" per unit of surface area (Huxley and Taylor, 1958).

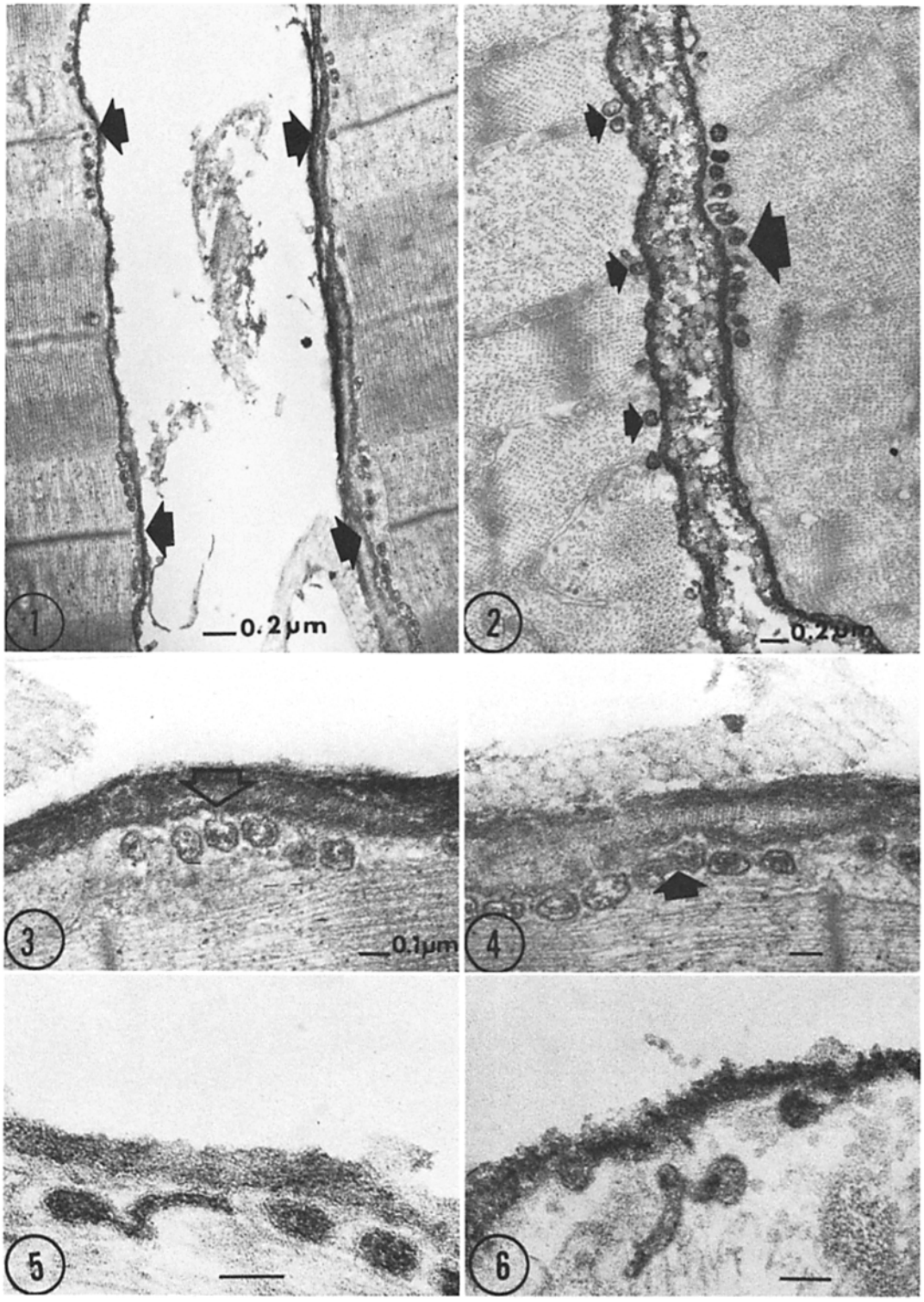
### *Association of the T Tubule with Caveolae*

Figs. 5 and 6 show that the caveolae are continuous with tubules below the plasma membrane. In order to investigate the possibility that these tubular profiles are part of the T system, we studied the morphology of the triads as they approach the surface of the fiber.

The triads, located deep inside the cytoplasm, display the organization previously described in the literature (Revel, 1962; Porter and Palade, 1957; Peachey, 1965; Franzini-Armstrong, 1970). These triads are comprised of a tubulous central element and two flattened cisternae as lateral elements. The membrane of the triads displays the unit membrane pattern and are about 7 nm thick (Robertson, 1959). The cytoplasmic leaflets of both unit membranes are separated by a gap about 10 nm wide, which is crossed by particles ("feet") of 16 to 18 nm diameter, spaced about 30 nm center-to-center (Franzini-Armstrong, 1970).

Where the triads approach the surface of the fiber the characteristics described above are maintained, except that the complete triad moves away from its close alignment with the Z line (Fig. 9). At about 150–200 nm from the sarcolemma, the array of particles connecting both cytoplasmic leaflets is lost. In this region, the T tubule opens into a round or slightly flattened profile, about 100 nm in diameter, similar to a caveola (Fig. 9).

We have observed 20 "face views" of triads near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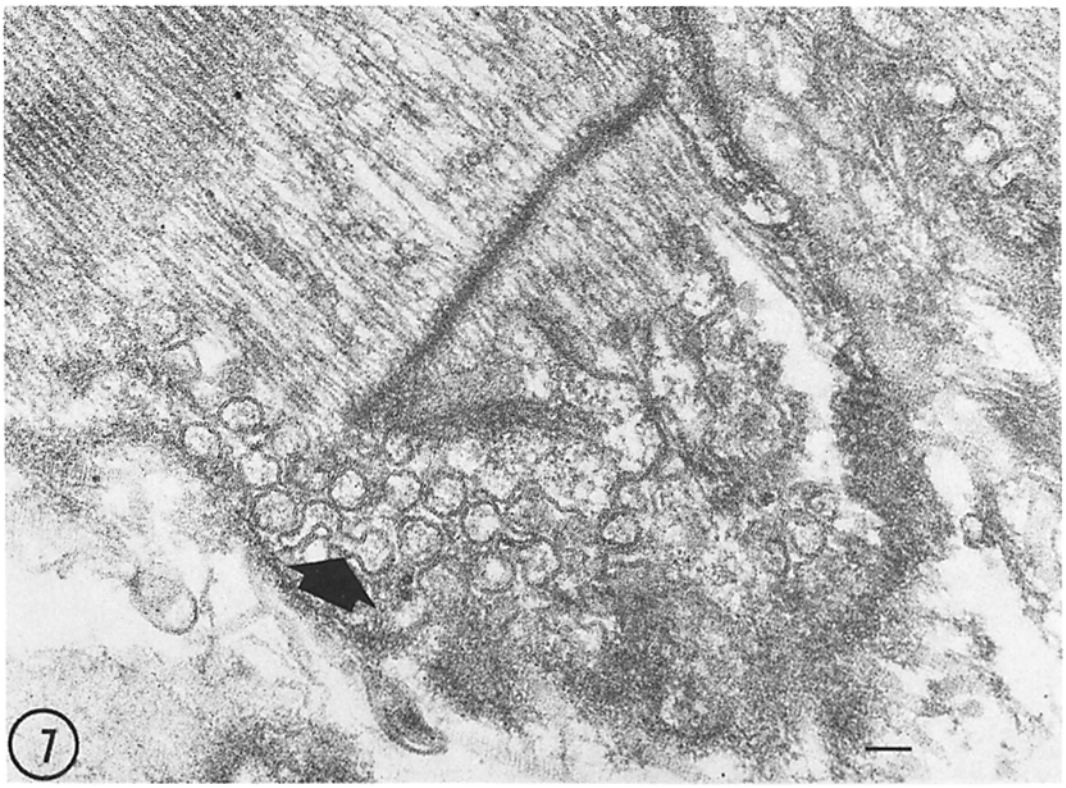


FIGURE 7 Longitudinal section of a muscle fiber at the myotendon junction, block-stained with uranyl acetate. Note the caveolae apparently located deep in the cytoplasm of the fiber. The arrow points to a connection between a deformed tubule and a caveola. × 60,000.

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FIGURE 1 Longitudinal section of two muscle fibers block-stained with ruthenium red. The arrow points to areas of high density of caveolae at both sides of the Z line. Note the spread of caveolae over the other bands of the sarcomere. × 25,000.

FIGURE 2 Transverse section of muscle fibers block-stained with ruthenium red. The large arrow points to a group of caveolae and the smaller arrows to areas where caveolae are scattered between the myofilaments. × 32,000.

FIGURE 3 Electron micrograph that shows the continuity between the caveolae membrane and the plasma membrane (arrow). Note the heavy deposits of ruthenium red in the basement lamina and in the lumen of the caveolae. × 52,000.

FIGURE 4 Longitudinal section of a muscle fiber. Note the elongated profile derived from the fusion between two adjacent caveolae. × 52,000.

FIGURE 5 Longitudinal section of a muscle fiber. At the I band a caveola is continuous with a thin tubule. × 100,000.

FIGURE 6 Transverse section of a muscle fiber showing an irregular, thin tubule connected to a caveola. × 85,000.

the surface of the fiber. In most of these views (17 out of 20) the T tubule distinctly opens into a caveola. In other cases, the T tubule maintains both the cylindrical shape and the array of particles (previously described for the triads) up to 2–4 nm from the plasma membrane.

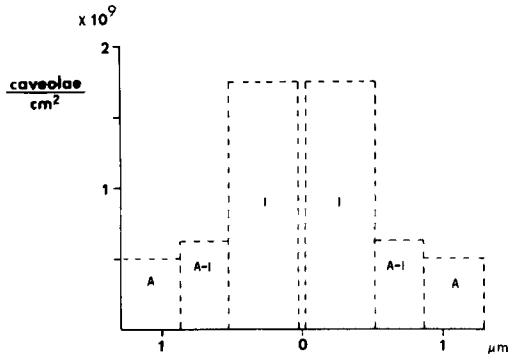


FIGURE 8 Histogram obtained by counting (350) caveolae. The count includes longitudinal views from three different experiments. The abscissa represents the length of the sarcomere surface. The greatest density is observed at the I band.

## DISCUSSION

It was suggested several years ago (Robertson, 1956; and for a complete historical background see Huxley, 1971) that the TTS present in the fiber cytoplasm may be the anatomical basis for the inward spread of the electrical signal that triggers the muscle contraction. Through the following years, morphologists tried to support this possibility by showing continuity between the plasma membrane and the tubule membrane. However, after years of careful analysis of the fiber surface, success has been reached only in a surprisingly small number of cases (Franzini-Armstrong and Porter, 1964; Birks, 1965; Walker and Schrodt, 1965; Hoyle et al., 1966; Page, 1968; and Ashhurst, 1969). An inevitable conclusion of these studies is that the T tubules very infrequently open "directly" to the outside.

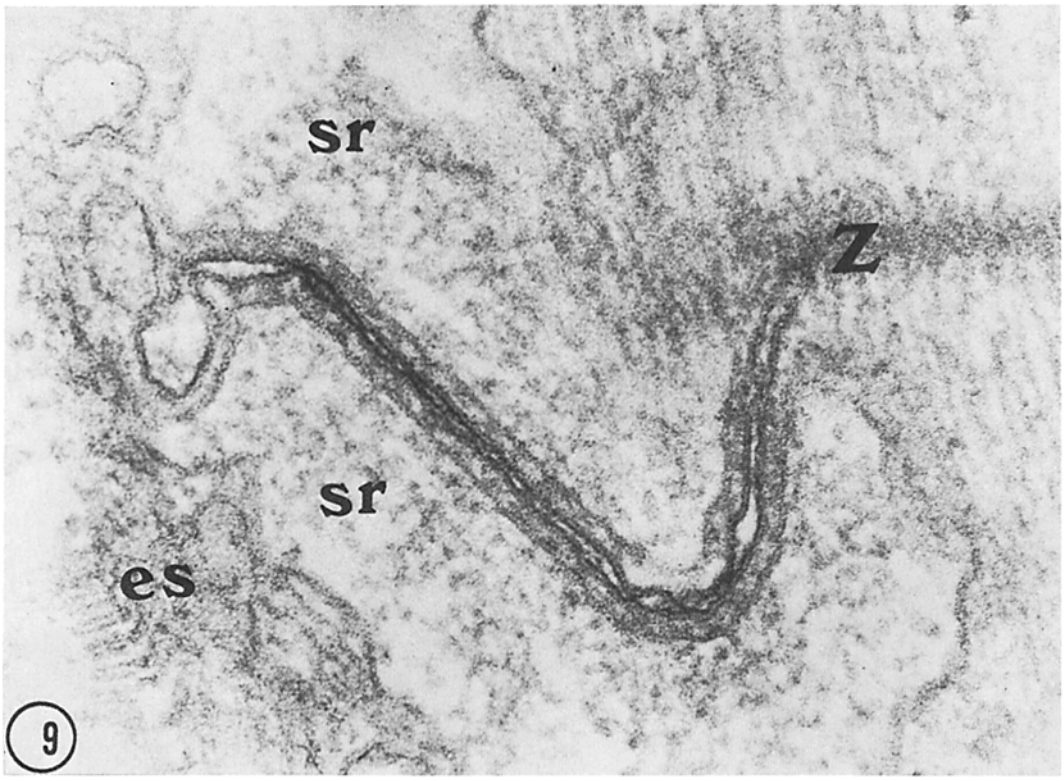


FIGURE 9 Triad region close to the surface of the fiber. Note that the main characteristics of the triad observed in Fig. 12 are maintained. However, the triad moves away from the Z line and then loses the particles associated with the cytoplasmic leaflets. *Z*, Z line; *ES*, extracellular space; *SR*, terminal cisternae of sarcoplasmic reticulum. This electron micrograph was selected from a tilting series. × 200,000.

The description of caveolae presented in this study may help to clarify the exact morphology of the connection between the TTS and the plasma membrane. It is clear from our results that T tubules can be seen opening to caveolae. We have found that at least 85% of the T tubules, in the intricate subsarcolemmal region, open to caveolae. Furthermore, other results obtained with extracellular tracers show that at least 90% of the caveolae open to the extracellular space and, very frequently, caveolae can be seen fusing among themselves. These observations suggest the following pattern of communication of the TTS with the outside: T tubules run in association with the SR as far as the subsarcolemmal region where they communicate with caveolae, which may also fuse to each other, and then the caveolae open to the extracellular space. That is, the caveolae seem to be the "openings" of the TTS of the semitendinosus muscle fibers.

In order for the caveolae to be the morphological representation of the openings of the TTS, they must be distributed preferentially in relation to the bands of the sarcomere. The localization and frequency of the openings of the T tubules to the surface of the fiber have been inferred from the results of Huxley and Taylor (1958) on "sensitive spots." [2] Huxley and Taylor found sensitive spots only when the pipette (2-$\mu$m tip diameter) overlapped the Z line of the sarcomeres of semitendinosus fibers. In agreement with these results we have shown that the highest density of caveolae occurs at the I band of the sarcomere. In other skeletal muscle fibers, where the triads face the junction between the A and I bands of the sarcomere, the largest density of caveolae also face the A–I junction (Rayns et al., 1968). Therefore, the highest density of caveolae seems to occur where the openings of the T tubules have been localized.

An independent piece of evidence concerning the morphological meaning of caveolae is found in the studies of differentiation of the T system (Ezerman and Ishikawa, 1967; Ishikawa, 1968). These studies suggest that caveolae are the first step in the development of the T tubules from the plasma membrane, in unmatured cultured muscle cells, supporting our conclusion that caveolae are part of the TTS.

In general, the results reported in this paper are consistent with the idea that caveolae are the openings of the TTS. However, one point on which we would like to make some comments is the number of caveolae communicating with each T tubule.

Our estimate of the number of caveolae gives a ratio of about 100 caveolae per sensitive spot. This surprisingly high ratio and the very high number of caveolae filled with the tracer (at least 90%), leads to the question of whether or not all the caveolae present in the subsarcolemmal region are continuous with the T tubules. A definitive answer cannot be given yet. However, our observations of a high number of interconnected caveolae at the I band strongly suggest that several caveolae are continuous with each T tubule, i.e. each T tubule may have several small openings to the outside.

Our results, as well as those reported in the literature on the morphology of the caveolae compartment, are consistent with the proposition that caveolae represent the openings of the T tubules to the extracellular space.

## SUMMARY

In frog skeletal muscle the region between the T system and the sarcolemma contains single rows of round profiles named caveolae. The largest number of caveolae occur at the I band although caveolae are spread over the rest of the sarcomere. Caveolae are continuous with the extracellular space, with adjacent caveolae, and with the T tubules.

These morphological characteristics lead us to propose the caveolae compartment as the opening of the T system to the extracellular space.

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[2] A sensitive spot is defined as the surface region where a localized reduction of membrane potential is able to induce a local contraction (Huxley, 1971).

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